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- (54) **DNA ENCODING TAURINE AND GABA TRANSPORTERS AND USES THEREOF**
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- (51) **Int. Cl.⁷** **C12N 15/12**
- (52) **U.S. Cl.** **435/320.1; 435/69.1; 435/252.3; 536/23.5**
- (58) **Field of Search** **435/69.1, 252.3, 435/320.1; 536/23.5**

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(57) **ABSTRACT**

This invention provides isolated nucleic acid molecules encoding two mammalian GABA transporters, a mammalian taurine transporter and two human GABA transporters; methods of isolating these nucleic acid molecules and vectors comprising such nucleic acid molecules as well as mammalian cells comprising such vectors. Nucleic acid probes for detecting nucleic acid molecules encoding mammalian or human GABA transporters, or mammalian or human taurine transporters; antisense oligonucleotides complementary to any sequences of a nucleic acid molecule which encodes a mammalian GABA or taurine transporter or human GABA or taurine transporter; and antibodies to the mammalian GABA or taurine transporters, or human GABA or taurine transporters are provided. Pharmaceutical compounds related to mammalian GABA or taurine transporters and to human GABA or taurine transporters are provided. Nonhuman transgenic animals which express DNA encoding normal or mutant mammalian GABA or taurine transporters, or normal or mutant human GABA or taurine transporters are provided. Further provided are methods for determining substrate binding, detecting expression, drug screening, and treatments for alleviating abnormalities associated with mammalian GABA or taurine transporters, or human GABA or taurine transporters.

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FIG. 1A

-120 -100 -80
GGCAGCGAACACAAGCGCATCCGGTAGAACGGAAAGAACAGGAATTGCAGAGTGACTTCA
-60 -40 -20
AGTCTCCATACGATTTACTACCCGGGTGACGGCAGTGACTCGACAGAGTAGCGGCTGCAG
0 20 40
GTGGGATGGATAACAGGGTCTCGGGAACGACCAGTAATGGAGAGACAAAGCCAGTGTGTC
M D N R V S G T T S N G E T K P V C P
60 80 100
CAGTCATGGAGAAGGTGGAGGAAGACGGTACCTTGAACGGGAGCAATGGACCAACAAGA
V M E K V E E D G T L E R E Q W T N K M
120 140 160
TGGAGTTCGTACTGTCAGTGGCGGGAGAGATCATTTGGCTTAGGCAACGTCTGGAGGTTTC
E F V L S V A G E I I G L G N V W R F P
180 200 220
CCTATCTCTGCTACAAGAACGGGGGAGGTGCCTTCTTTATTCCCTACCTCATCTTCCTAT
Y L C Y K N G G G A F F I P Y L I F L F
240 260 280
TTACCTGTGGCATTCTCTGTCTTCTTCCTGGAGACAGCGCTTGGCCAGTACACCAACCAGG
T C G I P V F F L E T A L G Q Y T N Q G
300 320 340
GAGGCATCACAGCCTGGAGGAAAATCTGTCCCATCTTCGAGGGCATCGGCTATGCCTCAC
G I T A W R K I C P I F E G I G Y A S Q
360 380 400
AGATGATCGTCAGCCTTCTCAATGTCTACTACATCGTTGTCCTGGCCTGGGCCCTCTTCT
M I V S L L N V Y Y I V V L A W A L F Y
420 440 460
ACCTCTTCAGCAGCTTCACCACTGACCTCCCCTGGGGTAGCTGCAGCCACGAGTGGAATA
L F S S F T T D L P W G S C S H E W N T

FIG. 1B

480 500 520
CAGAA[.]ACTGTGTG[.]GAGTTCCAGA[.]AAACCAACAAT[.]TCCCTGAATGTGACTTCTG[.]AGAATG
E N C V E F Q K T N N S L N V T S E N A

540 560 580
CCACATCCCCTGTCATCGAGTTCTGGGAGAGGCGAG[.]TCCTGAAGATCTCAGATGGCATCC
T S P V I E F W E R R V L K I S D G I Q

600 620 640
AGCACCTGGGGTCCCTGCGCTGGGAGCTGGTCCTGTGCCTCCTG[.]CTTGCCTGGATCATCT
H L G S L R W E L V L C L L L A W I I C

660 680 700
GCTATTTCTGCATCTGGAAAGGGGTCAAGTCCACAGGCAAGGTGGTGTACTTCACAGCTA[.]
Y F C I W K G V K S T G K V V Y F T A T

720 740 760
CTTTCCTTACCTCATGCTGGTGGTCCTGTTGATCCGAGGAGTA[.]AACTGCCTGGAGCAG
F P Y L M L V V L L I R G V T L P G A A

780 800 820
CCCAGGGAATTCAGTTT[.]TACCTGTACCCCAACATCACACGTCTGTGGGATCCCCAGGTGT
Q G I Q F Y L Y P N I T R L W D P Q V W

840 860 880
GGATGGATGCGGGC[.]ACCCAGATCTTCTTCTCCTTTGCCATCTGCCTGGGGTGCCTCACGG
M D A G T Q I F F S F A I C L G C L T A

900 920 940
CCCTGGGCAGCTACAACAAGTACCACAACA[.]ACTGCTACAGGGACTGCGTCGCCCTTTGCA
L G S Y N K Y H N N C Y R D C V A L C I

960 980 1000
TTCTCAACAGCAGCACCAGCTTCGTGGCCGGGTTTGCCATCTTCTCCATCCTGGGCTTCA[.]
L N S S T S F V A G F A I F S I L G F M

1020 1040 1060
TGTCTCAGGAGCAGGGCGTACCCATATCTGAGGTTGCTGAATCAGGCCCTGGCCTGGCAT[.]
S Q E Q G V P I S E V A E S G P G L A F

FIG. 1C

1080 1100 1120
TCATCGCCTACCCTCGAGCTGTGGTGATGTTACCTTTCTCGCCTTTGTGGGCCTGCTGTT
I A Y P R A V V M L P F S P L W A C C F

1140 1160 1180
TCTTCTTCATGGTGGTTCTCCTGGGACTAGACAGCCAGTTTGTGTGTGTAGAAAGCCTCG
F F M V V L L G L D S Q F V C V E S L V

1200 1220 1240
TGACAGCGCTGGTGGACATGTATCCCGGGGTGTTCCGTAAGAAGAACCGGAGGGAGATTC
T A L V D M Y P R V F R K K N R R E I L

1260 1280 1300
TCATCCTCATCGTGTCTGTCGTCTCTTTCTTCATCGGGCTCATTATGCTCACAGAGGGCG
I L I V S V V S F F I G L I M L T E G G

1320 1340 1360
GCATGTACGTGTTCCAGCTCTTCGACTACTATGCGGCCAGTGGCATTGTGTCTTCTCTTTG
M Y V F Q L F D Y Y A A S G M C L L F V

1380 1400 1420
TGGCCATCTTTGAGTCCCTCTGTGTGGCTTGGGTTTACGGAGCCAGCCGCTTCTATGACA
A I F E S L C V A W V Y G A S R F Y D N

1440 1460 1480
ACATTGAAGATATGATTGGGTACAAGCCGTGGCCTCTTATCAAATACTGTTGGCTCTTTT
I E D M I G Y K P W P L I K Y C W L F F

1500 1520 1540
TCACGCCAGCTGTGTGCCTGGCAACCTTCCTGTTCTCCCTGATCAAATACACGCCACTGA
T P A V C L A T F L F S L I K Y T P L T

1560 1580 1600
CCTACAACAAGAAGTACACATATCCATGGTGGGGGGATGCCCTGGGGTGGCTCCTAGCTC
Y N K K Y T Y P W W G D A L G W L L A L

1620 1640 1660
TGTCCTCCATGGTCTGCATTCTGCTGGAGCATCTACAAGCTCAGGACTCTCAAGGGCC
S S M V C I P A W S I Y K L R T L K G P

FIG. 1D

1680 1700 1720
CACTCAGAGAGAGACTTCGCCAGCTCGTGTGCCCCGGCTGAAGACCTTCCCCAGAAGAGCC
L R E R L R Q L V C P A E D L P Q K S Q

1740 1760 1780
AACCAGAGCTGACTTCTCCAGCGACACCGATGACGTCCCTCCTCAGGCTCACAGAAGTGG
P E L T S P A T P M T S L L R L T E L E

1800 1820 1840
AGTCTAACTGCTAGGGACGAGGCCTTTGACACACCTGCGAGTCTGTCTGTGGGGACAGCT
S N C

1860 1880 1900
ACAGACACAGAGGGCAGAACCACCCCTCCGTGCTGGGGCAGAGAGACA

FIG. 2A

-10

10

30

GGCGGCAGGGCGGCCATGACTGCGGAGCAAGCGCTGCCCCTGGGCAACGGGAAGGCGGCC

M T A E Q A L P L G N G K A A

50

70

90

GAGGAGGCGCGAGGGTCCGAGGCGCTGGGCGGCGGCGGCGGGGGCGCGGCGGGGACGCGC

E E A R G S E A L G G G G G G A A G T R

110

130

150

GAGGCGCGCGACAAGGCGGTCCACGAGCGCGGTCACTGGAACAACAAGGTGGAGTTCGTG

E A R D K A V H E R G H W N N K V E F V

170

190

210

TTGAGCGTAGCGGGAGAGATCATCGGTCTGGGCAACGTGTGGCGCTTCCCCTACCTGTGC

L S V A G E I I G L G N V W R F P Y L C

230

250

270

TACAAGAACGGCGGAGGGGCATTCCTGATTCCTTACGTGGTGTTTTCATCTGCTGTGGA

Y K N G G G A F L I P Y V V F F I C C G

290

310

330

ATCCCCGTCTTCTTCCTGGAAACGGCTCTGGGGCAGTTCACGAGCGAGGGCGGCATCACG

I P V F F L E T A L G Q F T S E G G I T

350

370

390

TGCTGGAGGAGAGTCTGTCCTTTATTTGAAGGCATCGGCTATGCAACACAGGTGATCGAG

C W R R V C P L F E G I G Y A T Q V I E

410

430

450

GCGCATCTCAATGTCTACTACATCATCCTGGCGTGGGCCATCTTCTACTTAAGCAAC

A H L N V Y Y I I I L A W A I F Y L S N

470

490

510

TGCTTCACCACCGAGCTCCCCTGGGCCACCTGTGGGCATGAGTGGAACACAGAGAAATGT

C F T T E L P W A T C G H E W N T E K C

530

550

570

GTGGAGTTCCAGAAGCTGAACTTCAGCAACTACAGTCATGTGTCCCTGCAGAACGCAACC

V E F Q K L N F S N Y S H V S L Q N A T

FIG. 2B

590 610 630
TCCC[.]CGGTCATGGAGTTCTGGGAACGCCGGGTCTTGGCTATATCTGATGGCATTGAACAC
S P V M E F W E R R V L A I S D G I E H

650 670 690
ATCGGGAACCTCCGATGGGAGCTGGCACTGTGTCTCCTGGCGGCTTGGACCATCTGCTAC
I G N L R W E L A L C L L A A W T I C Y

710 730 750
TTCTGCATCTGGAAGGGTACGAAGTCAACTGGAAAGGTCGTGTATGTCACTGCAACCTTC
F C I W K G T K S T G K V V Y V T A T F

770 790 810
CCCTACATCATGCTGCTGATCCTCCTGATCCGAGGGGTACAGTTGCCGGGTGCCTCGGAA
P Y I M L L I L L I R G V T L P G A S E

830 850 870
GGCATCAAGTTCTACCTGTACCCTGACCTCTCCCGGCTCTCTGATCCACAGGTGTGGGTG
G I K F Y L Y P D L S R L S D P Q V W V

890 910 930
GATGCTGGGACGCAGATCTTTTTCTCCTATGCCATCTGCCTGGGCTGCCTGACCGCTCTG
D A G T Q I F F S Y A I C L G C L T A L

950 970 990
GGGAGTTACAACA[.]ACTATAACAACA[.]ACTGCTACAGGGACTGTATTATGCTCTGCTGTCTG
G S Y N N Y N N N C Y R D C I M L C C L

1010 1030 1050
AACAGTGGCACCAGCTTCGTGGCTGGGTTTGCTATCTTCTCAGTCCTGGGCTTCATGGCG
N S G T S F V A G F A I F S V L G F M A

1070 1090 1110
TACGAGCAGGGCGTGCCTATTGCTGAGGTGGCAGAATCAGGTCCTGGACTGGCTTTCATC
Y E Q G V P I A E V A E S G P G L A F I

1130 1150 1170
GCCTACCCCAAGGCTGTCACTATGATGCCCCTGTCCCCATTGTGGGCCACCCTGTTCTTC
A Y P K A V T M M P L S P L W A T L F F

FIG. 2C

1190

1210

1230

ATGATGCTCATCTTCCTGGGCCTGGACAGTCAGTTTGTGTGTGTGGAGAGCCTTGTGACA

M M L I F L G L D S Q F V C V E S L V T

1250

1270

1290

GCCGTGGTTGACATGTACCCCAAGGTCTTCCGGCGGGGCTACCGGCGAGAACTGCTCATC

A V V D M Y P K V F R R G Y R R E L L I

1310

1330

1350

CTGGCCCTGTCCATTGTCTCTTATTTCCTAGGCCTGGTGATGCTGACAGAGGGAGGCATG

L A L S I V S Y F L G L V M L T E G G M

1370

1390

1410

TACATTTTCCAGCTTTTGGACTCATACGCCGCCAGTGGCATGTGCTTGCTCTTCGTGGCC

Y I F Q L F D S Y A A S G M C L L F V A

1430

1450

1470

ATCTTTGAGTGTGTCTGCATCGGCTGGGTGTATGGAAGTAACAGGTTCTATGACAATATT

I F E C V C I G W V Y G S N R F Y D N I

1490

1510

1530

GAGGACATGATTGGATAACCGGCCACTGTCACTCATCAAGTGGTGCTGGAAAGTTGTGACC

E D M I G Y R P L S L I K W C W K V V T

1550

1570

1590

CCTGGGATCTGTGCGGGCATCTTCATCTTCTTTCTGGTCAAGTACAAGCCGCTCAAGTAC

P G I C A G I F I F F L V K Y K P L K Y

1610

1630

1650

AACAATGTGTACACATATCCTGCTTGGGGCTACGGCATTGGCTGGCTCATGGCTCTGTCC

N N V Y T Y P A W G Y G I G W L M A L S

1670

1690

1710

TCCATGCTGTGCATCCCGCTCTGGATCTTCATCAAGCTGTGGAAGACAGAGGGCACCCTG

S M L C I P L W I F I K L W K T E G T L

1730

1750

1770

CCCGAGAAATTACAGAAGTTGACAGTCCCCAGCGCTGATCTGAAAATGAGGGGGCAAGCTT

P E K L Q K L T V P S A D L K M R G K L

1790 1810 1830
GGGGCCAGCCACGGATGGTGACCGTTAATGACTGTGAGGCCAAGGTCAAAGGCGACGGT
G A S P R M V T V N D C E A K V K G D G
1850 1870 1890
ACCATCTCTGCCATCACAGAGAAGGAGACGCACTTCTGATCCCCGCCAGCCACTTGGATG
T I S A I T E K E T H F
1910
TGTCTCCAGCCTTCCTTC

FIG. 3A

-120

-100

-80

GCCAACGCCGCGATCGCCGCCAATCCCGCCAGCCTCGGGCCGGGCCATCCGCTGTGGGCT

-60

-40

-20

TAGCCACCCAGATGCAGAGCCAGTGCCACAGCCTCTTCAGAGGAGCCTCTCAAGCAAAAC

0

20

40

GAGGAGATGGCCACCAAGGAGAAGCTTCAATGTCTGAAAGACTTCCACAAAGACATCCTG

M A T K E K L Q C L K D F H K D I L

60

80

100

AAGCCTTCTCCAGGGAAGAGCCCAGGCACGCGGCCTGAGGATGAGGCTGATGGGAAGCCC

K P S P G K S P G T R P E D E A D G K P

120

140

160

CCTCAGAGGGAGAAGTGGTCCAGCAAGATCGACTTTGTGCTGTCTGTGGCCGGAGGCTTC

P Q R E K W S S K I D F V L S V A G G F

180

200

220

GTGGGTTTGGGCAATGTCTGGCGTTTCCCGTACCTCTGCTACAAAATGGTGGAGGTGCA

V G L G N V W R F P Y L C Y K N G G G A

240

260

280

TTCCTCATACCGTATTTTATTTTCCTGTTTGGGAGCGGCCTGCCTGTGTTTTTCCTGGAG

F L I P Y F I F L F G S G L P V F F L E

300

320

340

GTCATCATAGGCCAGTACACCTCAGAAGGGGGGCATCACCTGCTGGGAGAAGATCTGCCCC

V I I G Q Y T S E G G I T C W E K I C P

360

380

400

TTGTTCTCTGGCATTGGCTACGCGTCCATCGTCATCGTGTCCCTCCTGAATGTGTACTAC

L F S G I G Y A S I V I V S L L N V Y Y

420

440

460

ATCGTCATCCTGGCCTGGGCCACATACTACCTATTCCAGTCTTTCAGAAGGATCTTCCC

I V I L A W A T Y Y L F Q S F Q K D L P

FIG. 3B

480 500 520
TGGGCCCACTGCAACCATAGCTGGAACACGCCACAGTGCATGGAGGACACCCTGCGTAGG
W A H C N H S W N T P Q C M E D T L R R

540 560 580
AACGAGAGTCACTGGGTCTCCCTTAGCGCCGCCAACTTCACTTCGCCTGTGATCGAGTTC
N E S H W V S L S A A N F T S P V I E F

600 620 640
TGGGAGCGCAACGTGCTCAGCCTGTCTCCGGAATCGACCACCCAGGCAGTCTGAAATGG
W E R N V L S L S S G I D H P G S L K W

660 680 700
GACCTCGCGCTCTGCCTCCTCTTAGTCTGGCTCGTCTGTTTTTCTGCATCTGGAAGGGT
D L A L C L L L V W L V C F F C I W K G

720 740 760
GTTCCGGTCCACAGGCAAGGTTGTCTACTTCACTGCTACTTTCCCGTTTGCCATGCTTCTG
V R S T G K V V Y F T A T F P F A M L L

780 800 820
GTGCTGCTGGTCCGTGGACTGACCCTGCCAGGTGCTGGTGAAGGCATCAAATTCTACCTG
V L L V R G L T L P G A G E G I K F Y L

840 860 880
TACCCTAACATCAGCCGCCTTGAGGACCCACAGGTGTGGATCGACGCTGGAACCTCAGATA
Y P N I S R L E D P Q V W I D A G T Q I

900 920 940
TTCTTTTCCTACGCTATCTGCCTGGGGGCCATGACCTCACTGGGAAGCTATAACAAGTAC
F F S Y A I C L G A M T S L G S Y N K Y

960 980 1000
AAGTATAACTCGTACAGGGACTGTATGCTGCTGGGATGCCTGAACAGTGGTACCAGTTTT
K Y N S Y R D C M L L G C L N S G T S F

1020 1040 1060
GTGTCTGGCTTCGCAATTTTTTCCATCCTGGGCTTCATGGCACAAGAGCAAGGGGTGGAC
V S G F A I F S I L G F M A Q E Q G V D

FIG. 3C

1080 1100 1120
ATTGCTGATGTGGCTGAGTCAGGTCCTGGCTTGGCCTTCATTGCCTACCCAAAAGCTGTG
I A D V A E S G P G L A F I A Y P K A V

1140 1160 1180
ACCATGATGCCGCTGCCACCTTTTGGTCCATTCTGTTTTTTATTATGCTCCTCTTGCTT
T M M P L P T F W S I L F F I M L L L L

1200 1220 1240
GGACTGGACAGCCAGTTTGTGAAGTCGAAGGACAGATCACATCCTTGGTTGATCTTTAC
G L D S Q F V E V E G Q I T S L V D L Y

1260 1280 1300
CCGTCCTTCCTAAGGAAGGGTTATCGTCGGGAAATCTTCATTGCCATCGTGTGCAGCATC
P S F L R K G Y R R E I F I A I V C S I

1320 1340 1360
AGCTACCTGCTGGGGCTGACGATGGTGACGGAGGGTGGCATGTATGTGTTTCAACTCTTT
S Y L L G L T M V T E G G M Y V F Q L F

1380 1400 1420
GACTACTATGCAGCTAGTGGTGTATGCCTTTTGTGGGTCGCATTCTTTGAATGTTTTGTT
D Y Y A A S G V C L L W V A F F E C F V

1440 1460 1480
ATTGCCCTGGATATATGGCGGTGATAACTTATATGACGGTATTGAGGACATGATCGGCTAT
I A W I Y G G D N L Y D G I E D M I G Y

1500 1520 1540
CGGCCTGGACCCTGGATGAAGTACAGCTGGGCTGTCATCACTCCAGCTCTCTGTGTTGGA
R P G P W M K Y S W A V I T P A L C V G

1560 1580 1600
TGTTTCATCTTCTCTCTCGTCAAGTATGTACCCCTGACCTACAACAAAGTCTACCGGTAC
C F I F S L V K Y V P L T Y N K V Y R Y

1620 1640 1660
CCTGATTGGGCAATCGGGCTGGGCTGGGGCCTGGCCCTTTCCTCCATGGTGTGTATCCCC
P D W A I G L G W G L A L S S M V C I P

FIG. 3D

168017001720

TTGGT[•]CATTGTCATC[•]CTCCTCTGCC[•]GGACGGAGGG[•]ACCGCTCCGCGTGAGAATCAAATAC[•]

L V I V I L L C R T E G P L R V R I K Y

174017601780

CTGATAACCCCCAGGGAGCCCAACCGCTGGGCTGTGGAGCGTGAAGGGGCTACGCCCTTT[•]

L I T P R E P N R W A V E R E G A T P F

180018201840

CACTCCAGAGCAACCCTCATGAACGG[•]TGCACTCATGAAACCCAGT[•]CACGTCATTGTGGAG[•]

H S R A T L M N G A L M K P S H V I V E

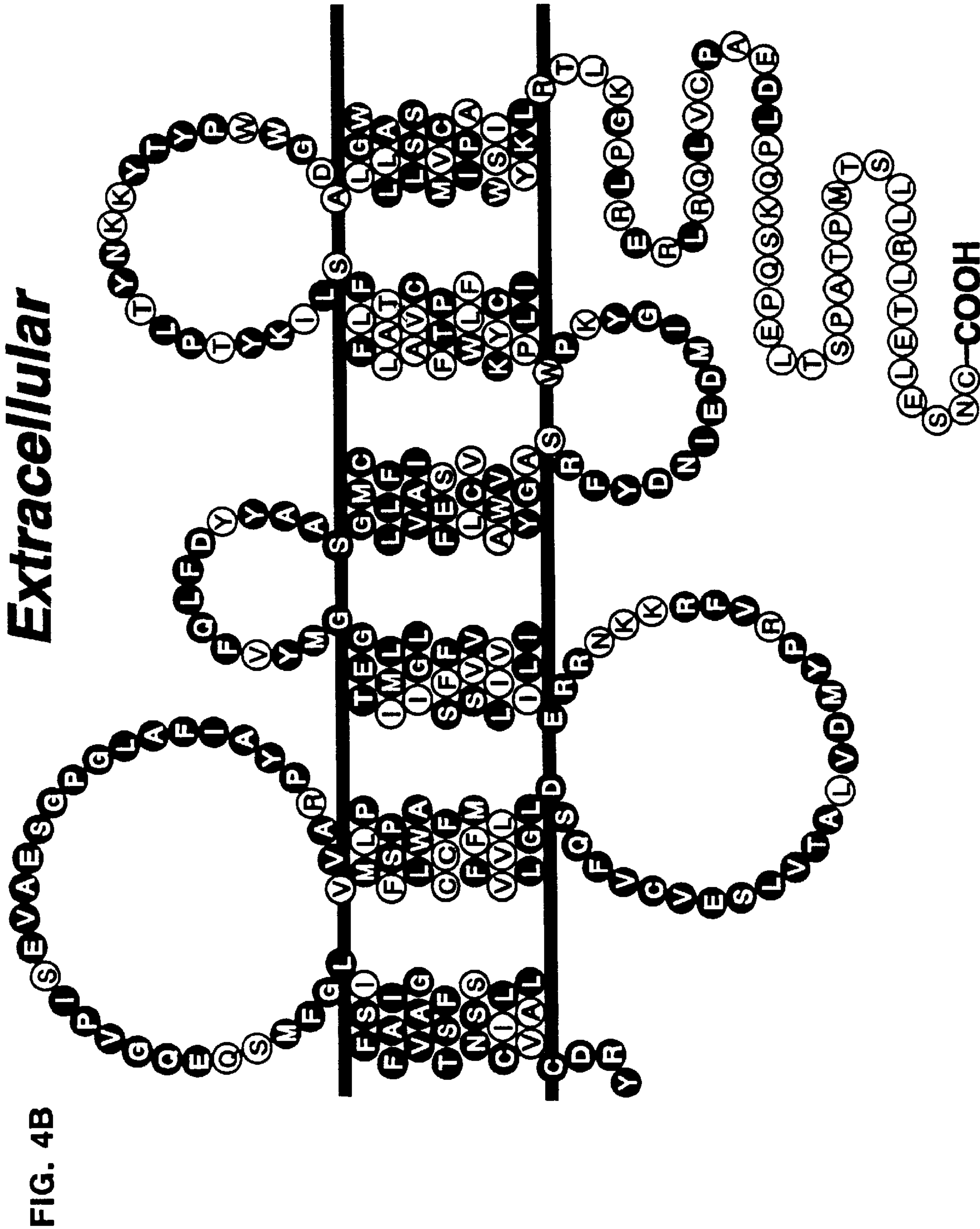
186018801900

ACCATGATGTGAGGTCCGGGCTGTGTGACCGGCGCCGCTTTCCTGCCGTTTACTAACCTT[•]

T M M

192019401960

AGATTCTCCTAGGACCAGGTTTACAGAGCTTTATATTTGTACTAGGATTTTTT[•]



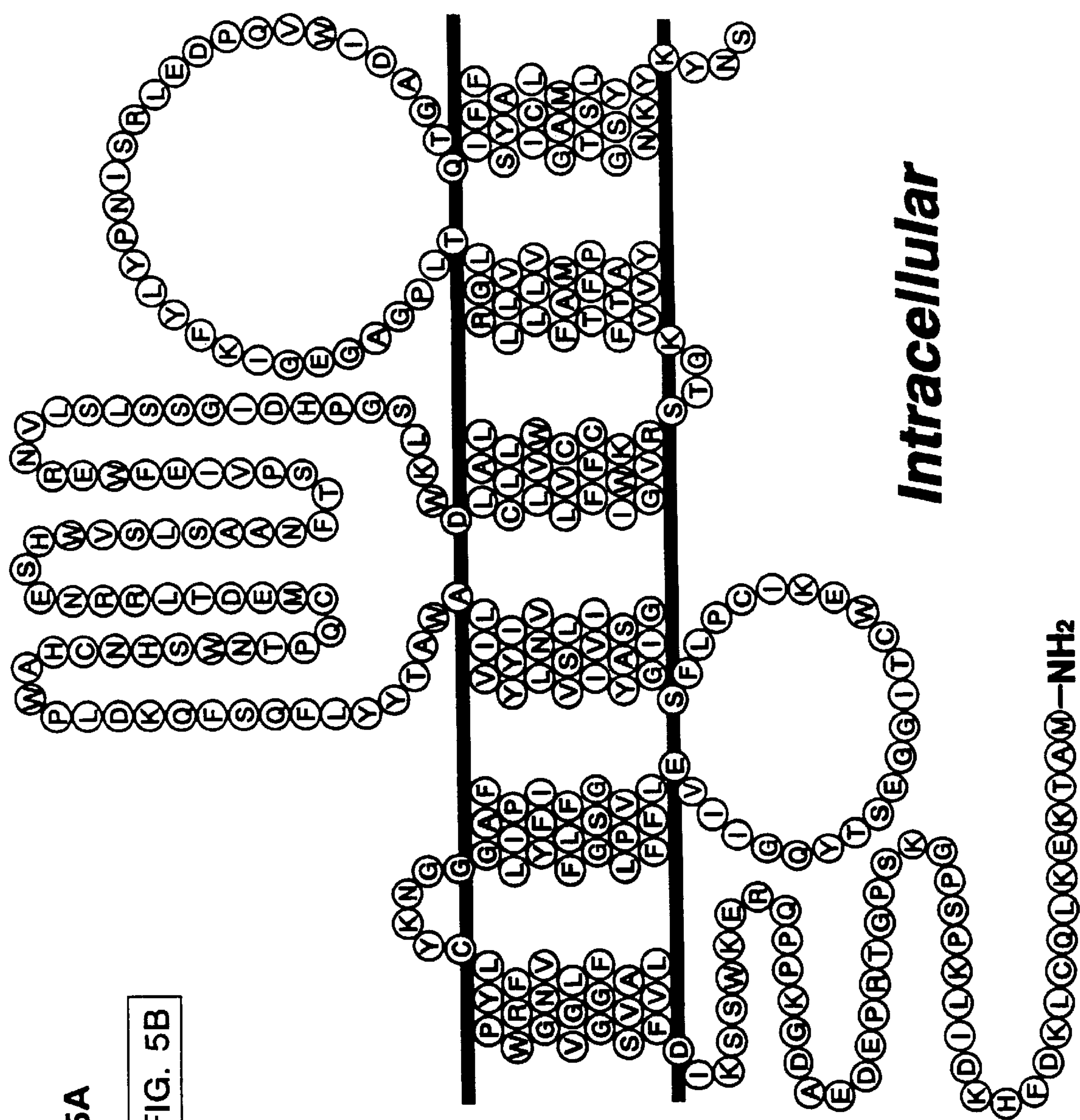


FIG. 5A

FIG. 5A | FIG. 5B

FIG. 6B

GAT-2		N	A	T	S	P	V	I	E	F	W	E	R	R	V	L	K	I	S	D	G	I	Q	H	L	G	S	L	R	W	E	L	V	L	C	L	L	A	W	I	I	G	Y	F	C	I	223	
GAT-3			N	A	T	S	P	V	I	E	F	W	E	R	R	V	L	K	I	S	D	G	I	Q	H	L	G	S	L	R	W	E	L	V	L	C	L	L	A	W	I	I	G	Y	F	C	I	238
GAT-1			N	A	T	S	P	V	I	E	F	W	E	R	R	V	L	K	I	S	D	G	I	Q	H	L	G	S	L	R	W	E	L	V	L	C	L	L	A	W	I	I	G	Y	F	C	I	229
Betaine			N	A	T	S	P	V	I	E	F	W	E	R	R	V	L	K	I	S	D	G	I	Q	H	L	G	S	L	R	W	E	L	V	L	C	L	L	A	W	I	I	G	Y	F	C	I	228
Glycine			N	A	T	S	P	V	I	E	F	W	E	R	R	V	L	K	I	S	D	G	I	Q	H	L	G	S	L	R	W	E	L	V	L	C	L	L	A	W	I	I	G	Y	F	C	I	237

GAT-2	W	K	G	V	K	G	T	K	K	S	I	G	K	K	V	V	Y	F	T	A	T	F	P	Y	L	M	L	V	V	L	L	I	R	G	V	I	L	L	I	F	F	L	L	I	Q	F	Y	L	Y	P	N	I	T	R	L	273
GAT-3	W	K	G	V	K	G	T	K	K	S	I	G	K	K	V	V	Y	F	T	A	T	F	P	Y	L	M	L	V	V	L	L	I	R	G	V	I	L	L	I	F	F	L	L	I	Q	F	Y	L	Y	P	N	I	T	R	L	288
GAT-1	W	K	G	V	K	G	T	K	K	S	I	G	K	K	V	V	Y	F	T	A	T	F	P	Y	L	M	L	V	V	L	L	I	R	G	V	I	L	L	I	F	F	L	L	I	Q	F	Y	L	Y	P	N	I	T	R	L	279
Betaine	W	K	G	V	K	G	T	K	K	S	I	G	K	K	V	V	Y	F	T	A	T	F	P	Y	L	M	L	V	V	L	L	I	R	G	V	I	L	L	I	F	F	L	L	I	Q	F	Y	L	Y	P	N	I	T	R	L	278
Glycine	I	R	G	V	K	S	I	G	K	V	V	Y	F	T	A	T	F	P	Y	L	M	L	V	V	L	M	L	V	V	L	L	I	R	G	V	I	L	L	I	F	F	L	L	I	Q	F	Y	L	Y	P	N	I	T	R	L	287

GAT-2	W	D	P	Q	V	W	M	D	A	G	T	Q	I	F	F	S	Y	A	I	G	L	G	C	L	L	I	A	L	G	S	Y	N	K	Y	H	N	N	N	C	Y	R	D	C	G	V	A	L	C	C	C	I	L	N	S	S	323
GAT-3	S	D	P	Q	V	W	M	D	A	G	T	Q	I	F	F	S	Y	A	I	G	L	G	C	L	L	I	A	L	G	S	Y	N	K	Y	H	N	N	N	C	Y	R	D	C	G	V	A	L	C	C	C	I	L	N	S	S	338
GAT-1	S	D	P	Q	V	W	M	D	A	G	T	Q	I	F	F	S	Y	A	I	G	L	G	C	L	L	I	A	L	G	S	Y	N	K	Y	H	N	N	N	C	Y	R	D	C	G	V	A	L	C	C	C	I	L	N	S	S	329
Betaine	K	D	P	Q	V	W	M	D	A	G	T	Q	I	F	F	S	Y	A	I	G	L	G	C	L	L	I	A	L	G	S	Y	N	K	Y	H	N	N	N	C	Y	R	D	C	G	V	A	L	C	C	C	I	L	N	S	S	328
Glycine	L	E	A	K	V	W	G	D	A	A	S	Q	I	F	F	S	Y	A	I	G	L	G	C	L	L	I	A	L	G	S	Y	N	K	Y	H	N	N	N	C	Y	R	D	C	G	V	A	L	C	C	C	I	L	N	S	S	337

FIG. 6C

GAT-2	T S F V A G F F A I F S S I L L G F F M S Q E E Q G G V P I I S E E V A A E E S S G P G L A F F I A Y P R A V V M L P F S P	373
GAT-3	T S F V A G F F A I F S S I L L G F F M S Q E E Q G G V P I I S E E V A A E E S S G P G L A F F I A Y P R A V V M L P F S P	388
GAT-1	T S F V A G F F A I F S S I L L G F F M S Q E E Q G G V P I I S E E V A A E E S S G P G L A F F I A Y P R A V V M L P F S P	379
Betaine	T S F V A G F F A I F S S I L L G F F M S Q E E Q G G V P I I S E E V A A E E S S G P G L A F F I A Y P R A V V M L P F S P	378
Glycine	T S F V A G F F A I F S S I L L G F F M S Q E E Q G G V P I I S E E V A A E E S S G P G L A F F I A Y P R A V V M L P F S P	387
GAT-2	L W A C C F F F F M V V L L L G L L D S Q F V C V E S L L V T A L V D M Y P R V F R R K K N R R R E E I L L I V	423
GAT-3	L W A C C F F F F M V V L L L G L L D S Q F V C V E S L L V T A L V D M Y P R V F R R K K N R R R E E I L L I V	438
GAT-1	L W A C C F F F F M V V L L L G L L D S Q F V C V E S L L V T A L V D M Y P R V F R R K K N R R R E E I L L I V	427
Betaine	L W A C C F F F F M V V L L L G L L D S Q F V C V E S L L V T A L V D M Y P R V F R R K K N R R R E E I L L I V	428
Glycine	L W A C C F F F F M V V L L L G L L D S Q F V C V E S L L V T A L V D M Y P R V F R R K K N R R R E E I L L I V	436
GAT-2	S V V S S F F I G L I M L L T E G G G M Y Y V F Q L F F D Y Y A A S S G M C L L F F V A I F E E S L C V A W V Y G A	473
GAT-3	S V V S S F F I G L I M L L T E G G G M Y Y V F Q L F F D Y Y A A S S G M C L L F F V A I F E E S L C V A W V Y G A	488
GAT-1	S V V S S F F I G L I M L L T E G G G M Y Y V F Q L F F D Y Y A A S S G M C L L F F V A I F E E S L C V A W V Y G A	477
Betaine	S V V S S F F I G L I M L L T E G G G M Y Y V F Q L F F D Y Y A A S S G M C L L F F V A I F E E S L C V A W V Y G A	478
Glycine	S V V S S F F I G L I M L L T E G G G M Y Y V F Q L F F D Y Y A A S S G M C L L F F V A I F E E S L C V A W V Y G A	485

FIG. 7A

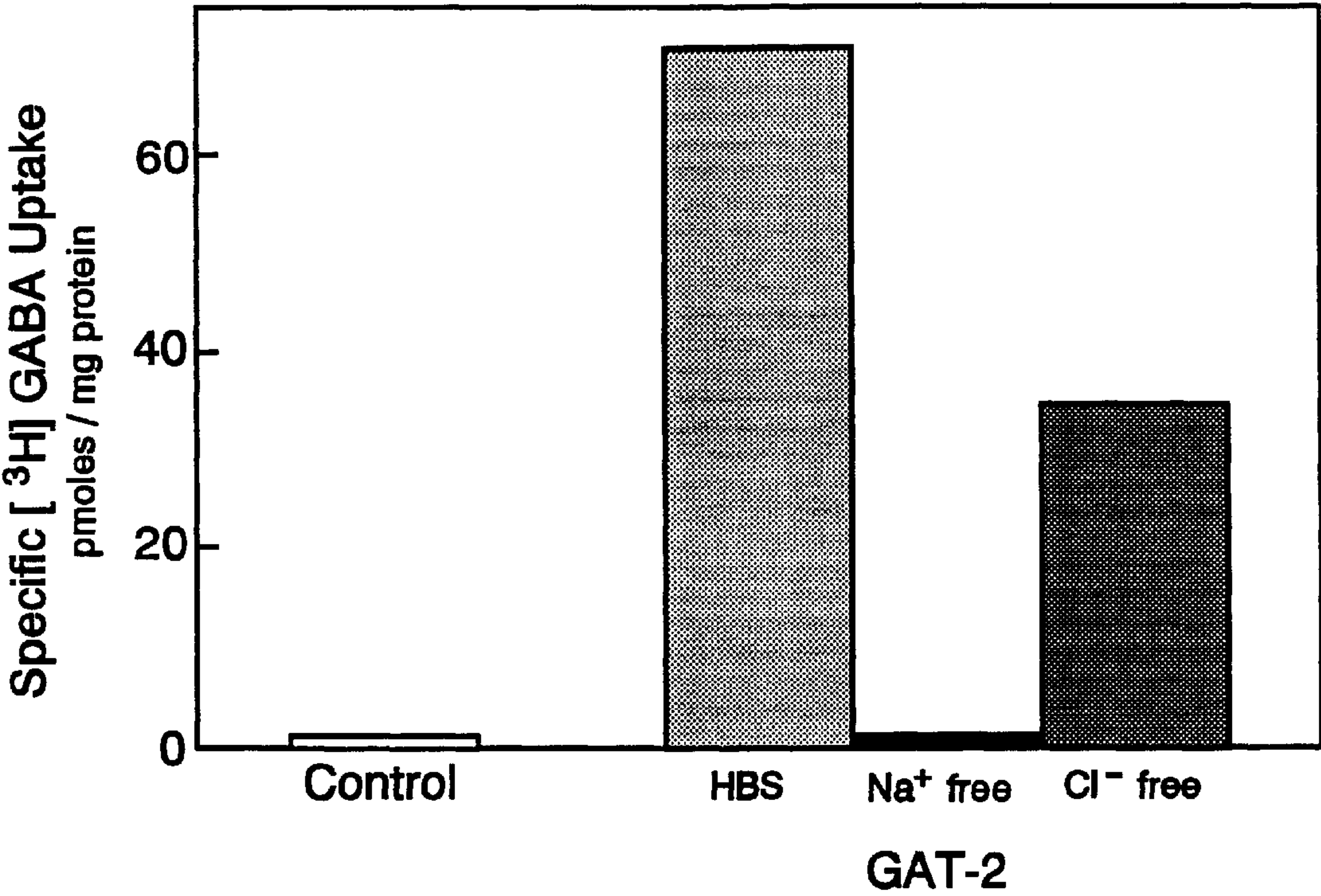


FIG. 7B

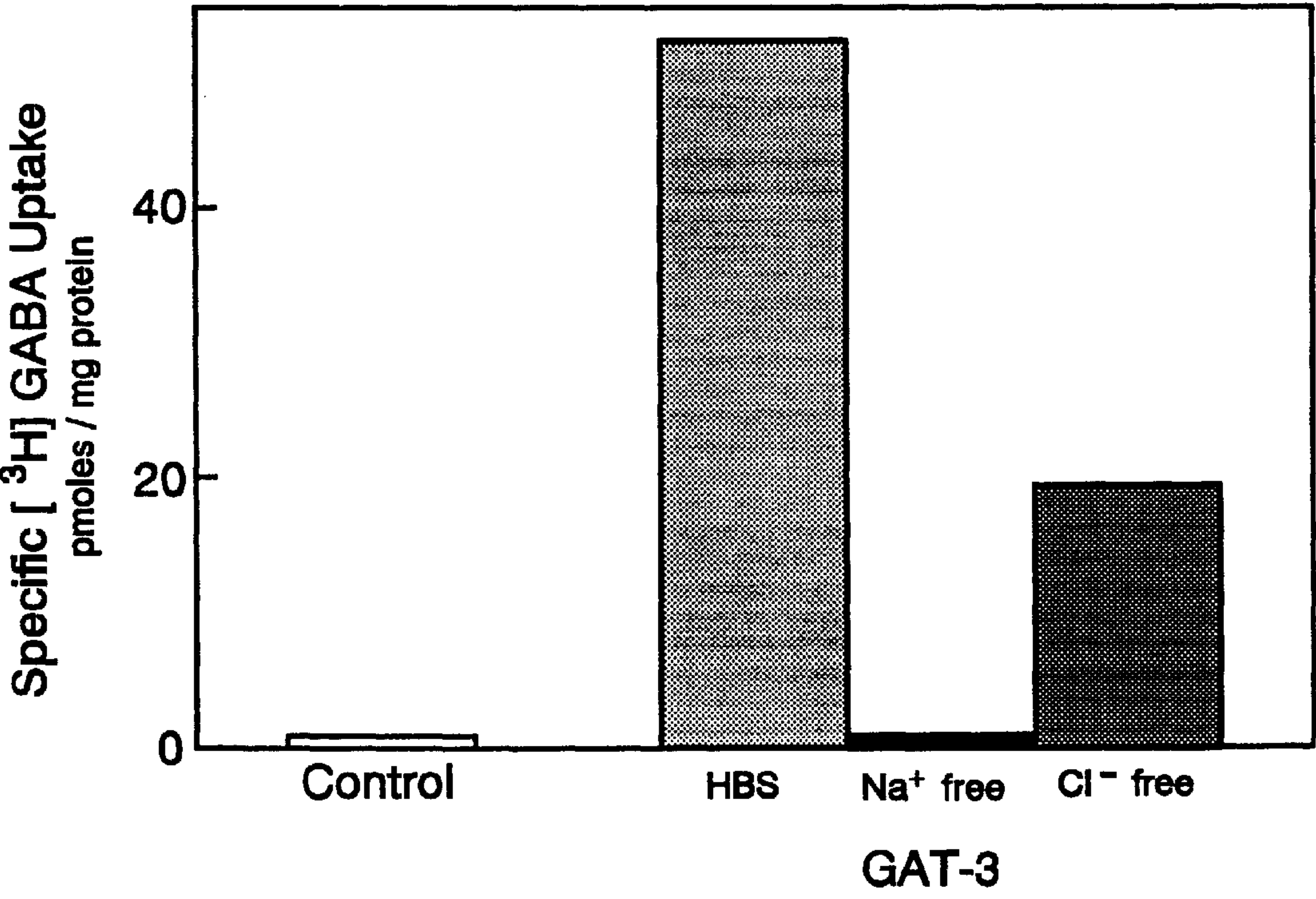


FIG. 8A

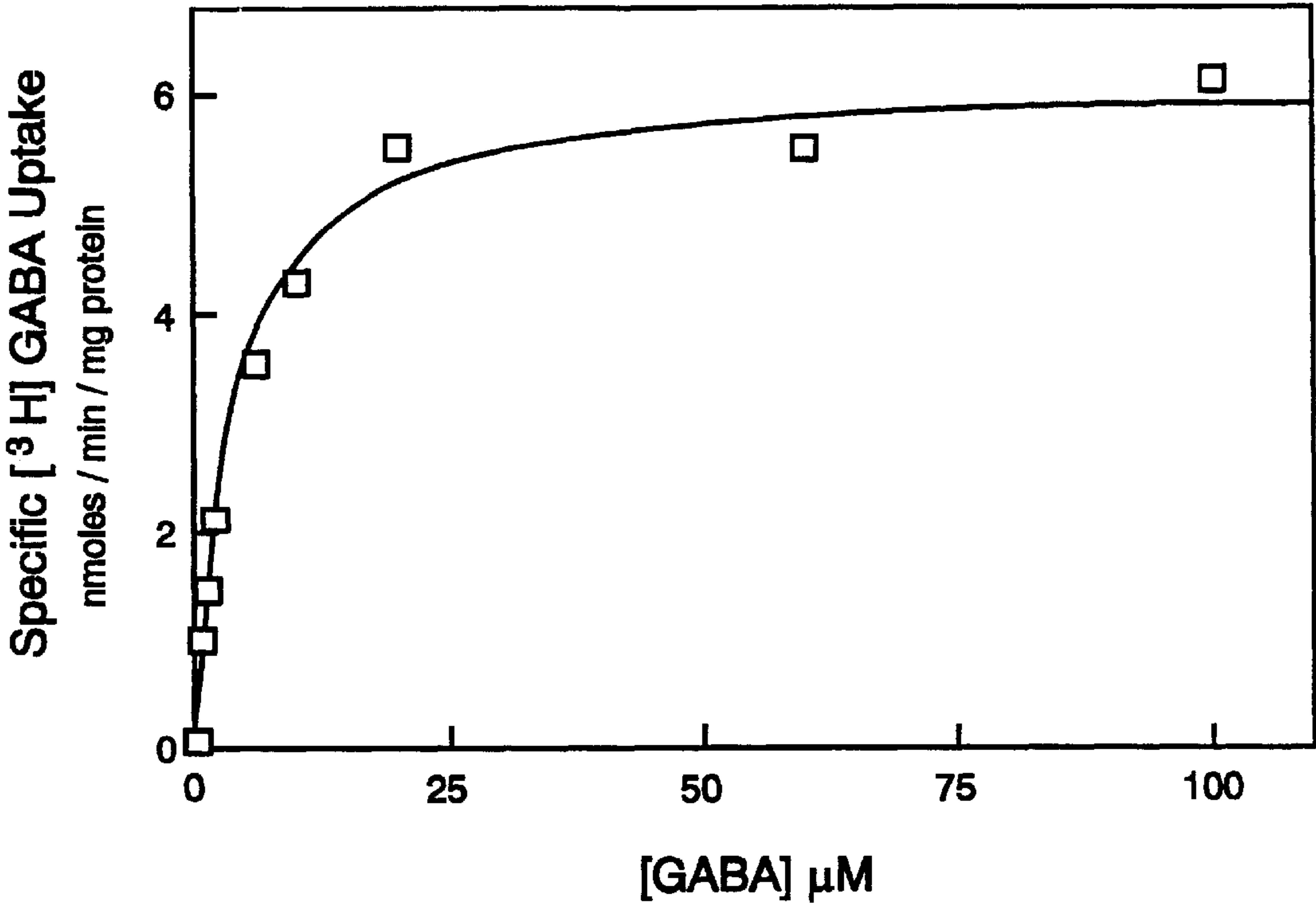


FIG. 8B

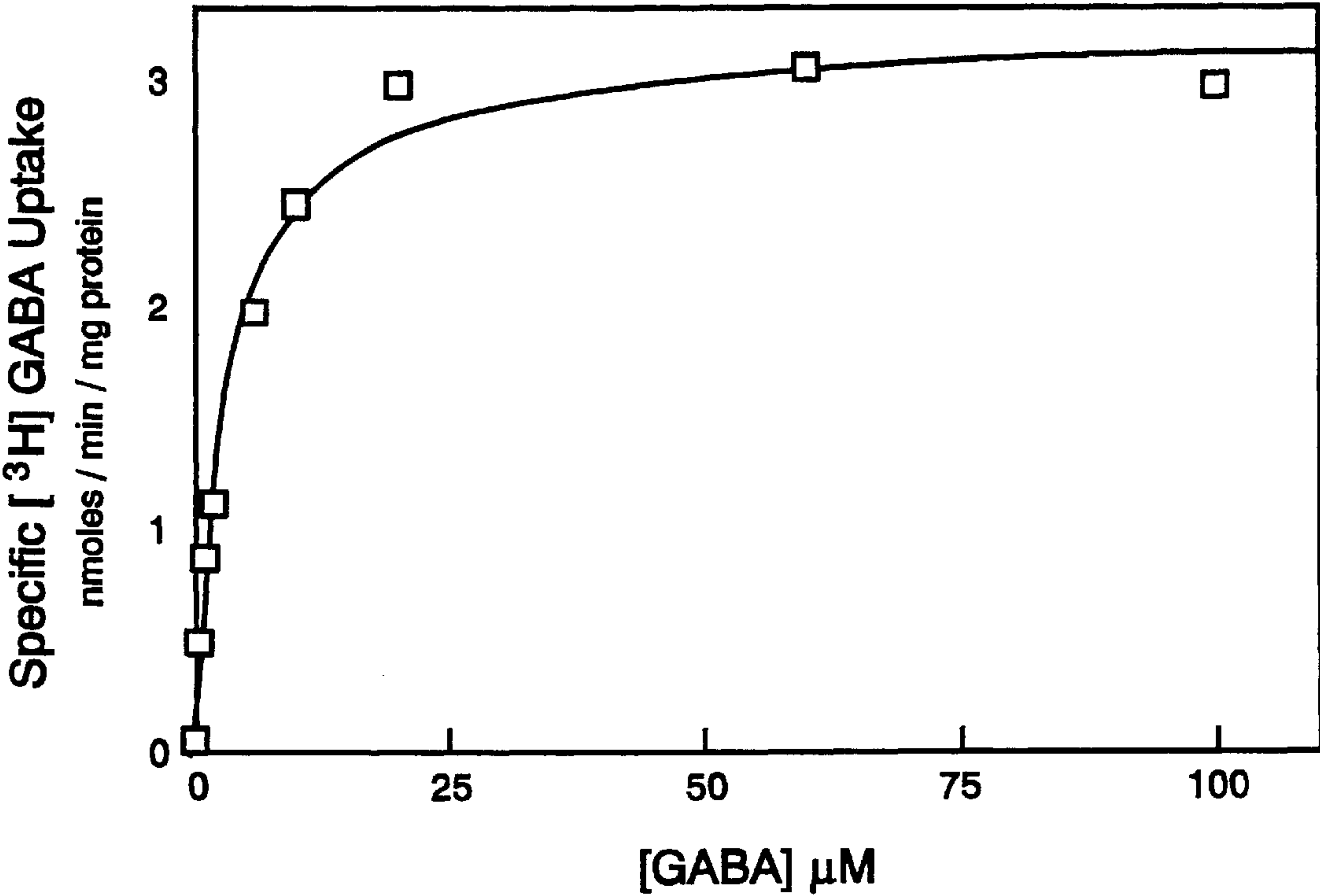


FIG. 9A

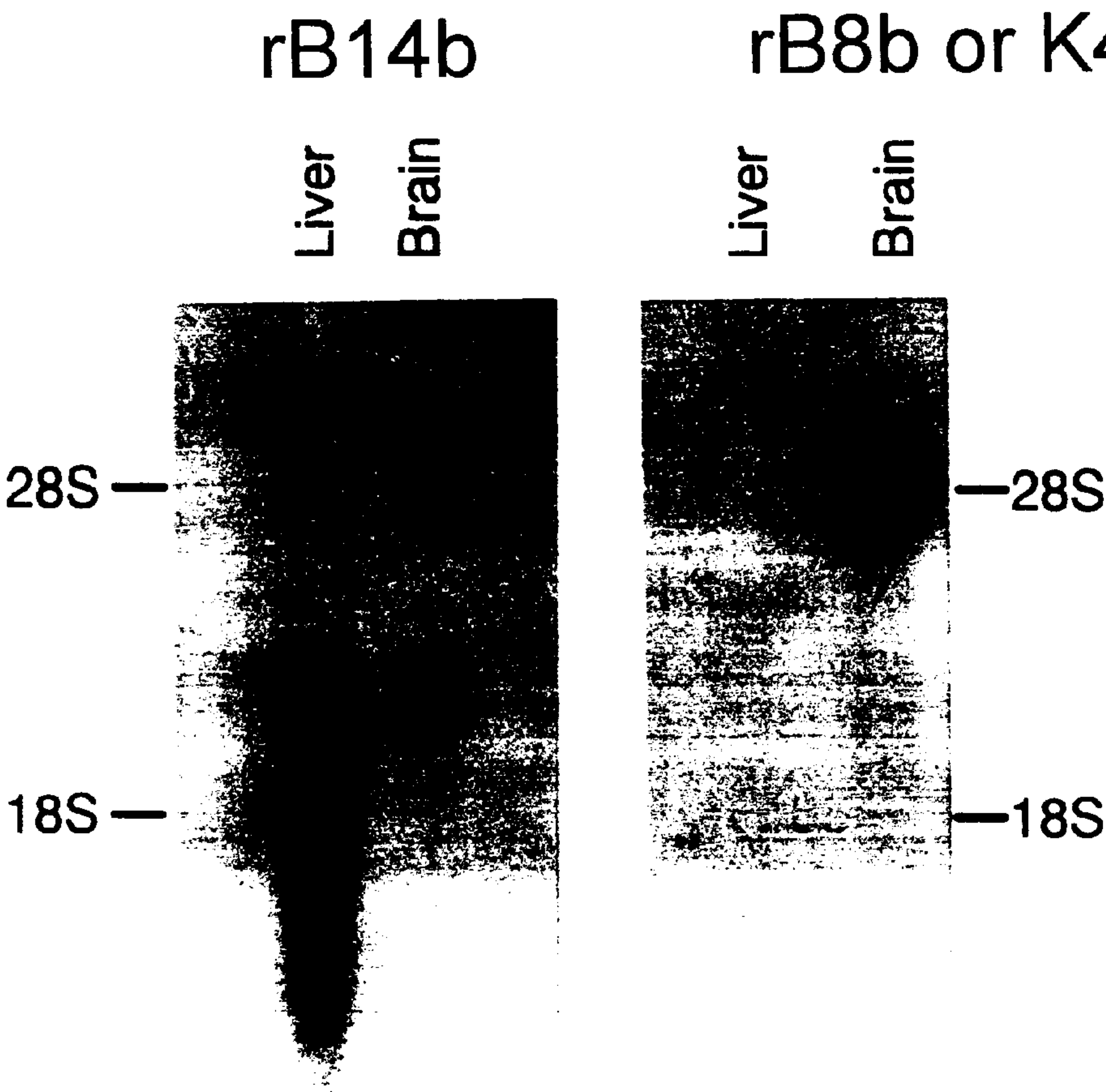


FIG. 9B

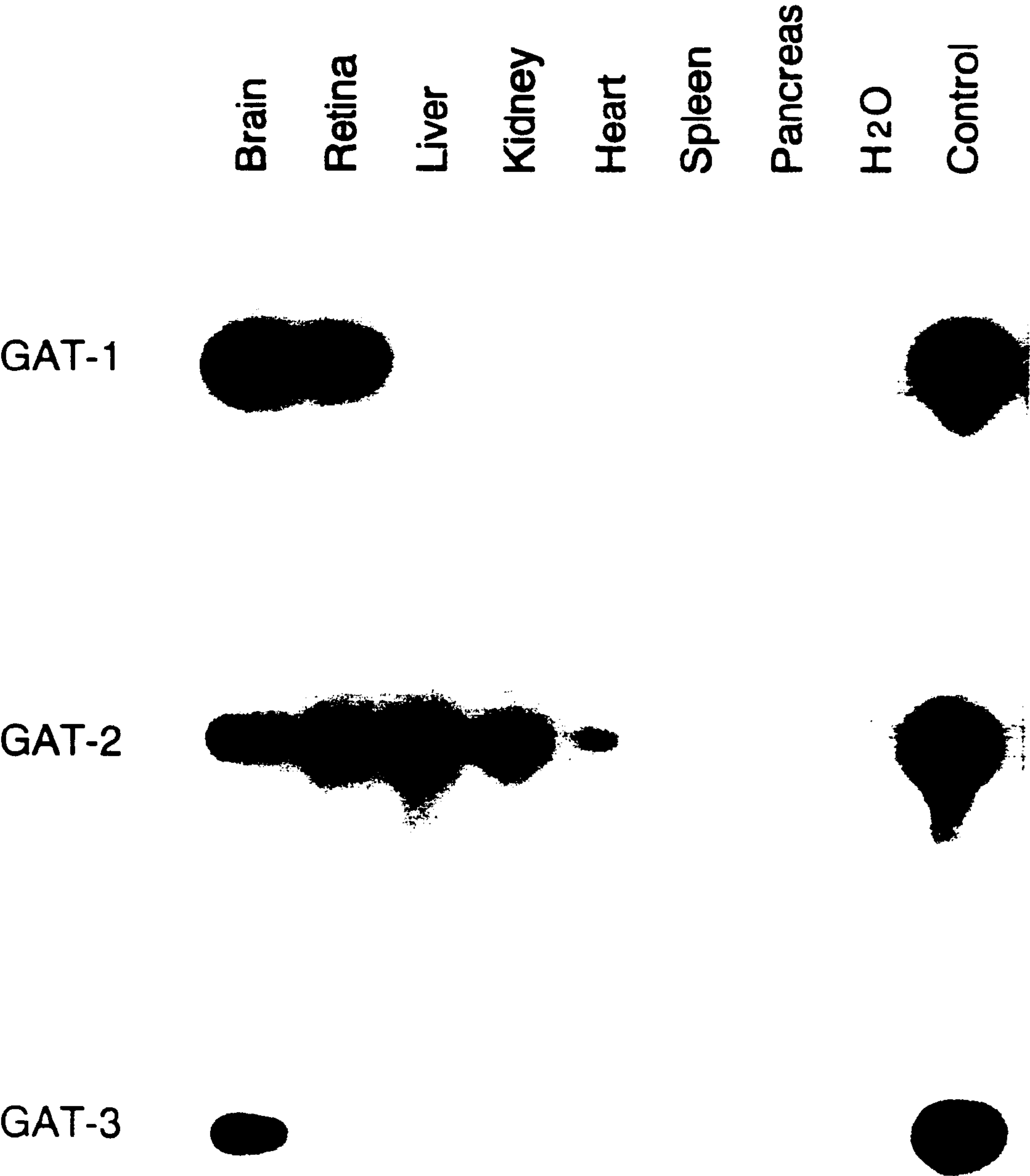


FIG. 10A

Taurine	M A T K E K L Q C L K D F H K D I L K P S P G K S P G T R P . . . E D E A D G K P P Q R E K	43
GAT-1	M A T D N S K V A D G Q I S T E V S E A P V A S D K P K T L V V K V Q K A G D L P D R D T	46
Betaine	M D R K V A V P E D G P P V V S W L P E E G E K L . . . D Q E G E D Q V K D R G Q	38
Glycine	M A V A H G P V A T S S P E Q N G A V P S E A T K K D Q N L T R G N	34
Taurine	W S S K I D F V L S V A G G F V G L G N V W R F P Y L C Y K N G G A F L I P Y F I F L F G S G L P	93
GAT-1	W K G R F D F L M S C V G Y A I G L G N V W R F P Y L C C G K N G G G A F L I P Y F L T L I F A G V P	96
Betaine	W T N K M E F V L S V A G E I I G L G N V W R F P Y L C C Y K N G G G A F F I P Y F I F T C G I P	88
Glycine	W G N Q I E F V L T S V G Y A V G L G N V W R F P Y L C Y R N G G G A F M F P Y F I M L V F C G I P	84
Taurine	V F F L E V I I G Q Y T S E G G I T C W E K I C P L F S G I G Y A S I V I V S L L N V Y Y I V I L A	143
GAT-1	L F L L E C S L G Q Y T S I G G L G V W . K L A P M F K G V G L A A V L S F W L N I Y Y I V I I S	145
Betaine	V F F L E V A L G Q Y T S Q G S V T A W R K I C P L L Q G I G L A S V V I E S Y L N I Y Y I I L A	138
Glycine	L F F M E L S F G Q F A S Q G C L G V W . R I S P M F K G V G Y G M M V V S T Y I G I Y Y N V V I C	133
Taurine	W A T Y Y L F Q S F Q K D L P W A H C N H S W N T P Q C . . . M E D T L R R N E S H W V S L S A .	189
GAT-1	W A I Y Y L Y N S F T T T L P W K Q C C D N P W N T D R C . . . F S N Y S L V N T T .	183
Betaine	W A L F Y L F S S F T S E L P W T T C T N T W N T E H C . . . M D . . . F L N H S G A R T A T S S E .	182
Glycine	I A F Y Y F F S S M T H V L P W A Y C N N P W N T P D C A G V L D A S N L T N G S R P T A L S G N L	183

FIG. 10B

Taurine		N F T S P V I E F W E R N V L S L S S G I D H P G S L K W D L A L C L L L V W L V C	IV -	231
GAT-1		N M T S A V V E F W E R N M H Q M T D G L D K P G Q I R W P L A I T L A I A W V L V		225
Betaine		N F T S P V M E F W E R R V L G I T S G I H D L G A L R W E L A L C L L L A W L I C		224
Glycine	S H L F N Y T L Q R T S P S E E Y W R L Y V L K L S D D I G D F G E V R L P L L G C L G V S W V V V			233
Taurine	F F C I W K G V R S T G K V V Y F T A T F P F A M L L V L L V R G L T L P G A G E G I K F Y L Y P N	V		281
GAT-1	Y F C I W K G V G W T G K V V Y F S A T Y P Y I M L I I L F F R G V T L P G A K E G I L F Y I T P N			275
Betaine	Y F C I W K G V K T T G K V V Y F T A T F P Y L M L V I L L I R G I T L P G A Y Q G V I Y Y L K P D			274
Glycine	F L C L I R G V K S S G K V V Y F T A T F P Y V V L T I L F V R G V T L E G A F T G I M Y Y L T P K			283
Taurine	I S R L E D P Q V W I D A G T Q I F F S Y A I C L G A M T S L G S Y N K Y K Y N S Y R D C M L L G C	VI		331
GAT-1	F R K L S D S E V W L D A A T Q I F F S Y G L G L G S L I A L G S Y N S F H N N V Y R D S I I V C C			325
Betaine	L L R L K D P Q V W M D A G T Q I F F S F A I C Q G C L T A L G S Y N K Y H N N C Y R D S I A L C F			324
Glycine	W D K I L E A K V W G D A A S Q I F Y S L G C A W G G L I T M A S Y N K F H N N C Y R D S V I I S I			333
Taurine	L N S G T S F V S G F A I F S I L G F M A Q E Q G V D I A D V A E S G P G L A F I A Y P K A V T M M	VII		381
GAT-1	I N S C T S M F A G F V I F S I V G F M A H V T K R S I A D V A A S G P G L A F L A Y P E A V T Q L			375
Betaine	L N S A T S F A A G F V V F S I L G F M A Q E Q G L P I S E V A E S G P G L A F I A F P K A V T M M			374
Glycine	T N C A T S V Y A G F V I F S I L G F M A N H L G V D V S R V A D H G P G L A F V A Y P E A L T L L			383

FIG. 11

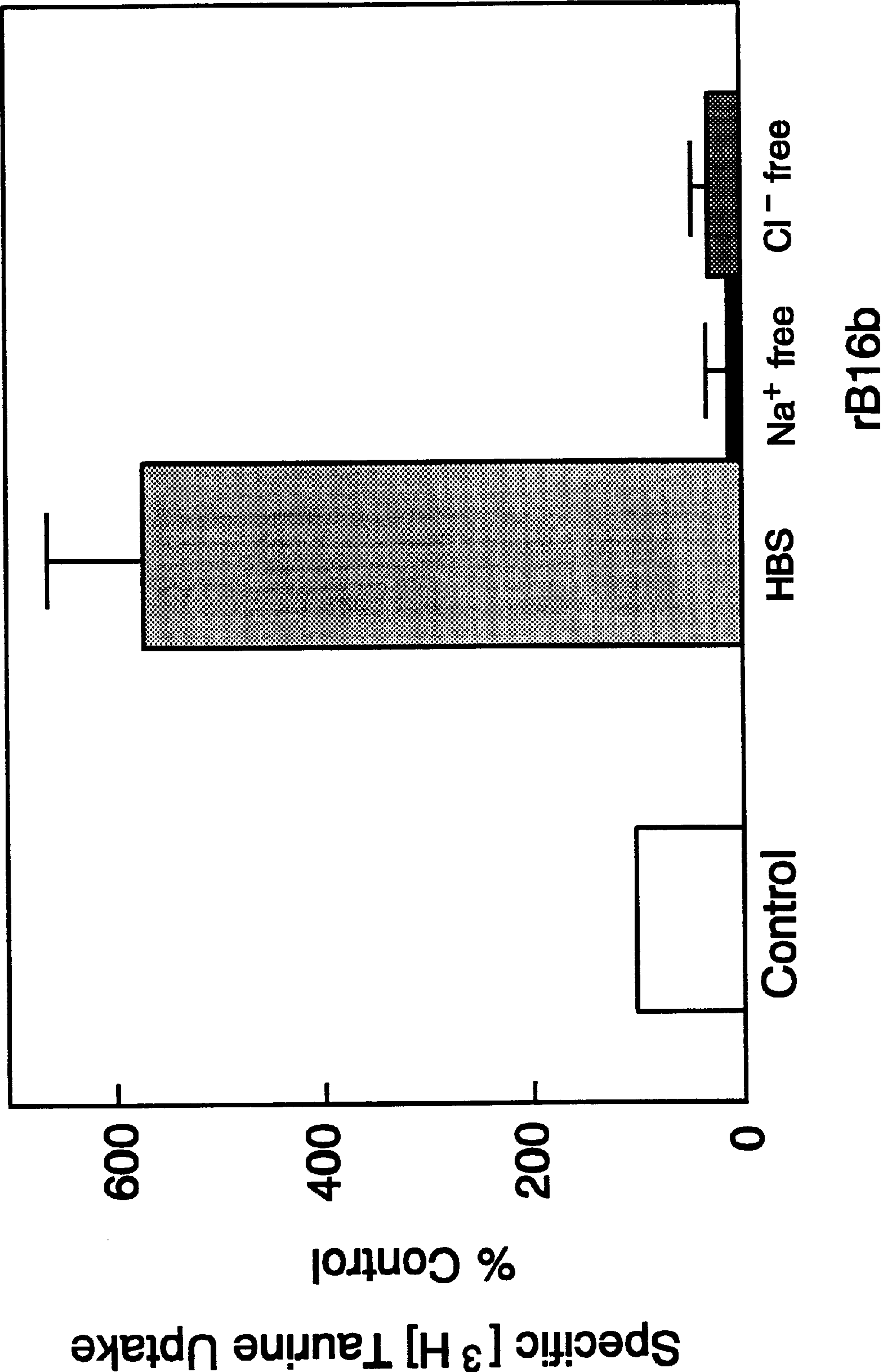


FIG. 12

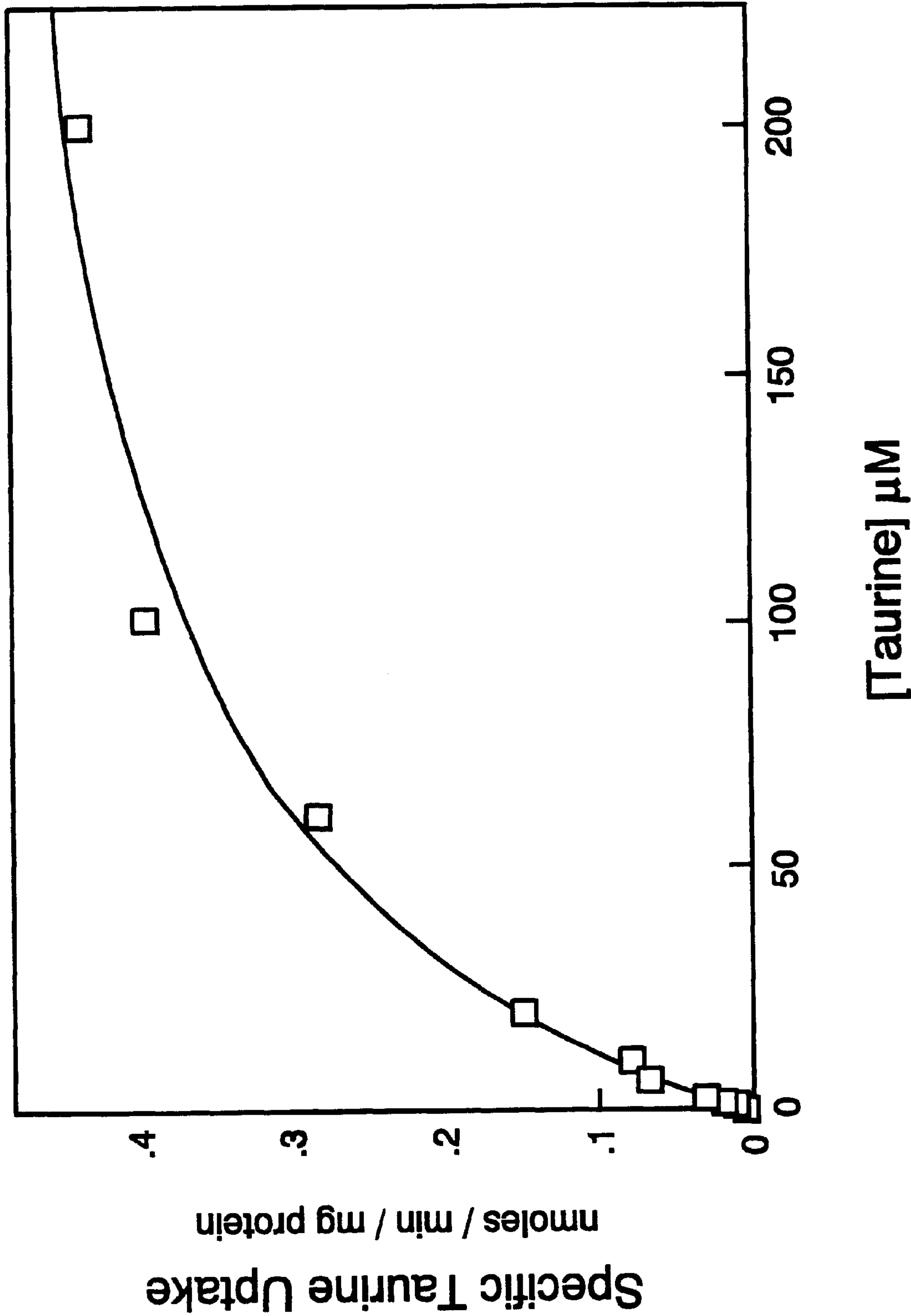


FIG. 13A

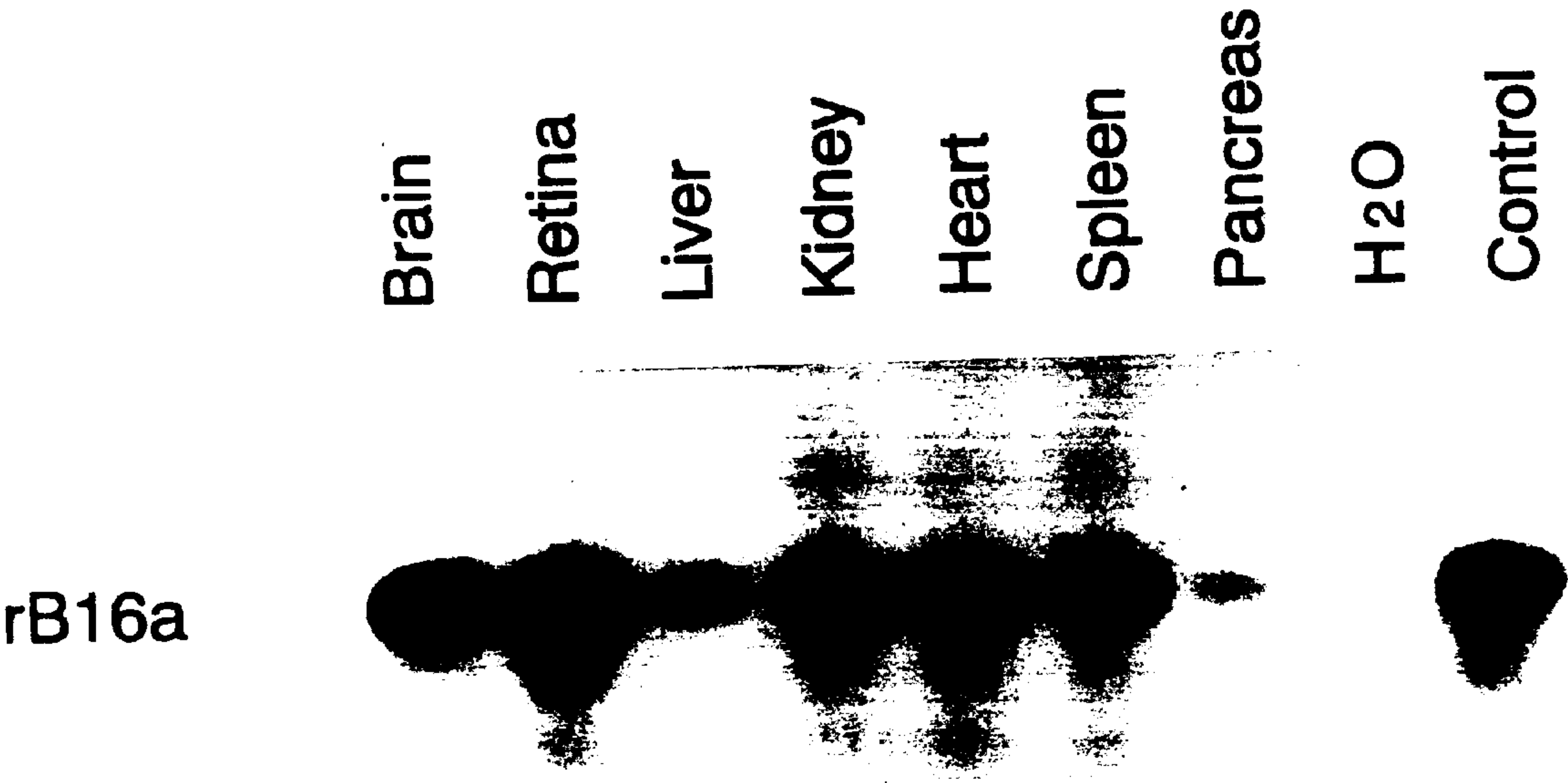


FIG. 14A

10 30 50
CTGGCTTTCATCGCTTACCCGCGGGCTGTGGTGATGCTGCCCTTCTCTCCTCTCTGGGCC
L A F I A Y P R A V V M L P F S P L W A

70 90 110
TGCTGTTTCTTCTTCATGGTCGTTCTCCTGGGACTGGATAGCCAGTTTGTGTGTGTAGAA
C C F F F M V V L L G L D S Q F V C V E

130 150 170
AGCCTGGTGACAGCGCTGGTGGACATGTACCCTCACGTGTTCCGCAAGAAGAACCGGAGG
S L V T A L V D M Y P H V F R K K N R R

190 210 230
GAAGTCCTCATCCTTGGAGTATCTGTCGTCTCCTTCCTTGTGGGGCTGATCATGCTCACA
E V L I L G V S V V S F L V G L I M L T

250 270 290
GAGGGCGGAATGTACGTGTTCCAGCTCTTTGACTACTATGCGGCCAGTGGCATGTGCCTC
E G G M Y V F Q L F D Y Y A A S G M C L

310 330 350
CTGTTTCGTGGCCATCTTCGAGTCCCTCTGTGTGGCTTGGGTTTACGGAGCCAAGCGCTTC
L F V A I F E S L C V A W V Y G A K R F

370 390 410
TACGACAACATCGAAGACATGATTGGGTACAGGCCATGGCCTCTTATCAAATACTGTTGG
Y D N I E D M I G Y R P W P L I K Y C W

430 450 470
CTCTTCCTCACACCAGCTGTGTGCACAGCCACCTTTCTCTTCTCCCTGATAAAGTACACT
L F L T P A V C T A T F L F S L I K Y T

490 510 530
CCGCTGACCTACAACAAGAAGTACACGTACCCGTGGTGGGGCGATGCCCTGGGCTGGCTC
P L T Y N K K Y T Y P W W G D A L G W L

550 570 590
CTGGCTCTGTCCTCCATGGTCTGCATTCCTGCCTGGAGCCTCTACAGACTCGGAACCCTC
L A L S S M V C I P A W S L Y R L G T L

FIG. 14B

610

630

650

AAGGGCCCCTTCAGAGAGAGAATCCGTCAGCTCATGTGCCCAGCCGAGGACCTGCCCCAG

K G P F R E R I R Q L M C P A E D L P Q

670

690

710

CGGAACCCAGCAGGACCCTCGGCTCCCGCCACCCCCAGGACCTCACTGCTCAGACTCAC

R N P A G P S A P A T P R T S L L R L T

730

750

770

GAGCTAGAGTCTCACTGCTAGGGGGCAGGCCCTTGGATGGTGCCTGTGTGCCTGGCCTTG

E L E S H C

790

810

830

GGGATGGCTGTGGAGGGAACGTGGCAGAAGCAGCCCCATGTGCTTCCCTGCCCCCGACCT

850

870

890

GGAGTGGATAAGACAAGAGGGGTATTTTGGAGTCCACCTGCTGAGCTGGAGGCCTCCAC

910

930

950

TGCAACTTTTCAGCTCAGGGGTTGTTGAACAGATGTGAAAGGCCAGTGCCAAGAGTGTCC

970

990

1010

CTCTGAGACCCTTGGGAAGCTGGGTGGGGGCTGGTAGGTGGGGCGAGACTTGCTGGCTTC

1030

1050

GGGCCCTCTCATCCTTCATTCCATTAATCC

FIG. 15A

-30 -10 10
AGCCGGGCGGCGCACGAGGCAGCCAGCGCGGCCATGACGGCGGAGAAGGCGCTGCCCCT
M T A E K A L P L

30 50 70
GGGCAATGGGAAGGCTGCTGAGGAGGCGCGGGAGTCCGAGGCGCCGGGTGGCGGCTGCAG
G N G K A A E E A R E S E A P G G G C S

90 110 130
CAGCGGGGGCGCGGCGCCCGCGCGCCACCCGCGCGTCAAGCGCGACAAGGCGGTCCACGA
S G G A A P A R H P R V K R D K A V H E

150 170 190
GCGCGGCCACTGGAACAACAAGGTGGAGTTTCGTGCTGAGCGTG GCCGGGGAGATCATTTGG
R G H W N N K V E F V L S V A G E I I G

210 230 250
GCTGGGCAACGTGTGGCGCTTCCCCTACCTGTGCTACAAGAACGGAGGAGGGGCATT CCT
L G N V W R F P Y L C Y K N G G G A F L

270 290 310
GATTCCTACGTGGTGTTTTTTTATTTGCTGTGGAATTCCTGTTTTTTTTCCTGGAGACAGC
I P Y V V F F I C C G I P V F F L E T A

330 350 370
TCTGGGGCAGTTCACAAGTGAAGGTGGCATTACGTGTTGGAGGAAAGTTTGCCCTTTATT
L G Q F T S E G G I T C W R K V C P L F

390 410 430
TGAAGGCATTGGCTATGCAACACAGGTGATTGAGGCCCATCTGAATGTGTACTACATCAT
E G I G Y A T Q V I E A H L N V Y Y I I

450 470 490
CATCCTGGCATGGGCCATTTTTTACCTGAGCAACTGCTTCACTACTGAGCTACCCTGGGC
I L A W A I F Y L S N C F T T E L P W A

510 530 550
TACCTGTGGGCATGAGTGGAACACAGAGAATTGTGTGGAGTTCCAGAAACTGAATGTGAG
T C G H E W N T E N C V E F Q K L N V S

FIG. 15B

570590610

CAACTACAGCCATGTGTCTCTGCAGAATGCCACCTCCCCTGTCATGGAGTTTGGGAGCA

N Y S H V S L Q N A T S P V M E F W E H

630650670

CCGGGTCCTGGCCATCTCTGACGGGATCGAGCACATCGGGAACCTTCGCTGGGAGCTGGC

R V L A I S D G I E H I G N L R W E L A

690710730

CTTGTGTCTCTTGGCAGCCTGGACCATCTGTTACTTCTGTATCTGGAAGGGGACCAAGTC

L C L L A A W T I C Y F C I W K G T K S

750770790

TACAGGAAAGGTTGTATACGTGACTGCGACATTCCCCTACATCATGCTGCTGATCCTCCT

T G K V V Y V T A T F P Y I M L L I L L

810830850

GATACGAGGGGTCACGTTGCCCGGGGCCTCAGAGGGCATCAAGTTCTACTTGTACCCTGA

I R G V T L P G A S E G I K F Y L Y P D

870890910

CCTCTCCCGGCTCTCCGACCCCCAGGTCTGGGTAGATGCTGGAACGCAGATCTTTTCTC

L S R L S D P Q V W V D A G T Q I F F S

930950970

CTATGCCATTTGCCTGGGCTGTCTGACCGCTCTGGGAAGTTATAACAATTATAACAACAA

Y A I C L G C L T A L G S Y N N Y N N N

99010101030

CTGCTACAGGGACTGCATCATGCTCTGTTGCCTGAACAGCGGCACCAGCTTCGTGGCTGG

C Y R D C I M L C C L N S G T S F V A G

105010701090

GTTTGCCATCTTCTCAGTCCTGGGTTTTATGGCGTACGAGCAGGGGGTACCCATTGCTGA

F A I F S V L G F M A Y E Q G V P I A E

111011301150

GGTGGCAGAGTCAGGCCCGGCCTGGCCTTTATTGCGTACCCCAAGGCGGTCACCATGAT

V A E S G P G L A F I A Y P K A V T M M

FIG. 15C

1170 1190 1210
GCCTCTCTCCCCGCTGTGGGCCACCTTGTTCTTCATGATGCTCÂTCTTCCTGGGCTGGA
P L S P L W A T L F F M M L I F L G L D

1230 1250 1270
CAGCCAGTTTGTGTGTGTGGAAAGCCTGGTGACCGCCGTGGTGGACATGTACCCCAAGGT
S Q F V C V E S L V T A V V D M Y P K V

1290 1310 1330
TTTCCGGAGGGGTÂTACCGGCGGGÂGCTGCTCATCCTAGCCTTGCTGTATTATCTCCTATTT
F R R G Y R R E L L I L A L S V I S Y F

1350 1370 1390
TCTGGGCCTCGTGÂTGTTAACAGÂGGGTGGCATGTACATCTTCCAGCTCTTTGÂCTCCTA
L G L V M L T E G G M Y I F Q L F D S Y

1410 1430 1450
TGCCGCCAGTGGGÂTGTCCTTCTCTTCGTGGCCATCTTTGAGTGCATCTGCATCGGCTG
A A S G M C L L F V A I F E C I C I G W

1470 1490 1510
GGTGTATGGAAGCAACCGGTTCTÂTGATAACATTGAAGACATGÂTTGGCTACCGGCCACC
V Y G S N R F Y D N I E D M I G Y R P P

1530 1550 1570
GTCGCTCATTAAGTGGTGCTGGATGATCATGACCCCTGGGATCTGCGCGGGGATCTTCAT
S L I K W C W M I M T P G I C A G I F I

1590 1610 1630
CTTCTTCTTGATCAAGTACAAGCCACTCAAGTACAACAACATCTACACCTACCCAGCCTG
F F L I K Y K P L K Y N N I Y T Y P A W

1650 1670 1690
GGGCTATGGCATTGGCTGGCTCATGGCCCTGTCCTCCATGCTCTGCATCCCGCTCTGGAT
G Y G I G W L M A L S S M L C I P L W I

1710 1730 1750
CTGCATCACAGTGTGGAAGACGGÂGGGGACACTGCCCGAGAACTCCAGAAGTTGACGAC
C I T V W K T E G T L P E K L Q K L T T

FIG. 15D

177017901810

CCCAGCACAGATCTGAAAATGCGGGGCAAGCTTGGGGTGAGCCACGGATGGTGACAGT

PSTDLKMRGLGVSPRMVTV

183018501870

TAATGACTGTGATGCCAAACTCAAGAGTGACGGGACCATCGCAGCCATCACAGAGAAGGA

NDCKLKS DGTIAAIT EKE

189019101930

GACGCACTTCTGAGCGGCCACCAGCCATCTGGGGCTCTTCTTCCTTTCTTCCCCCGTGT

THF*

1950

ATGTAAATGAA

FIG. 16

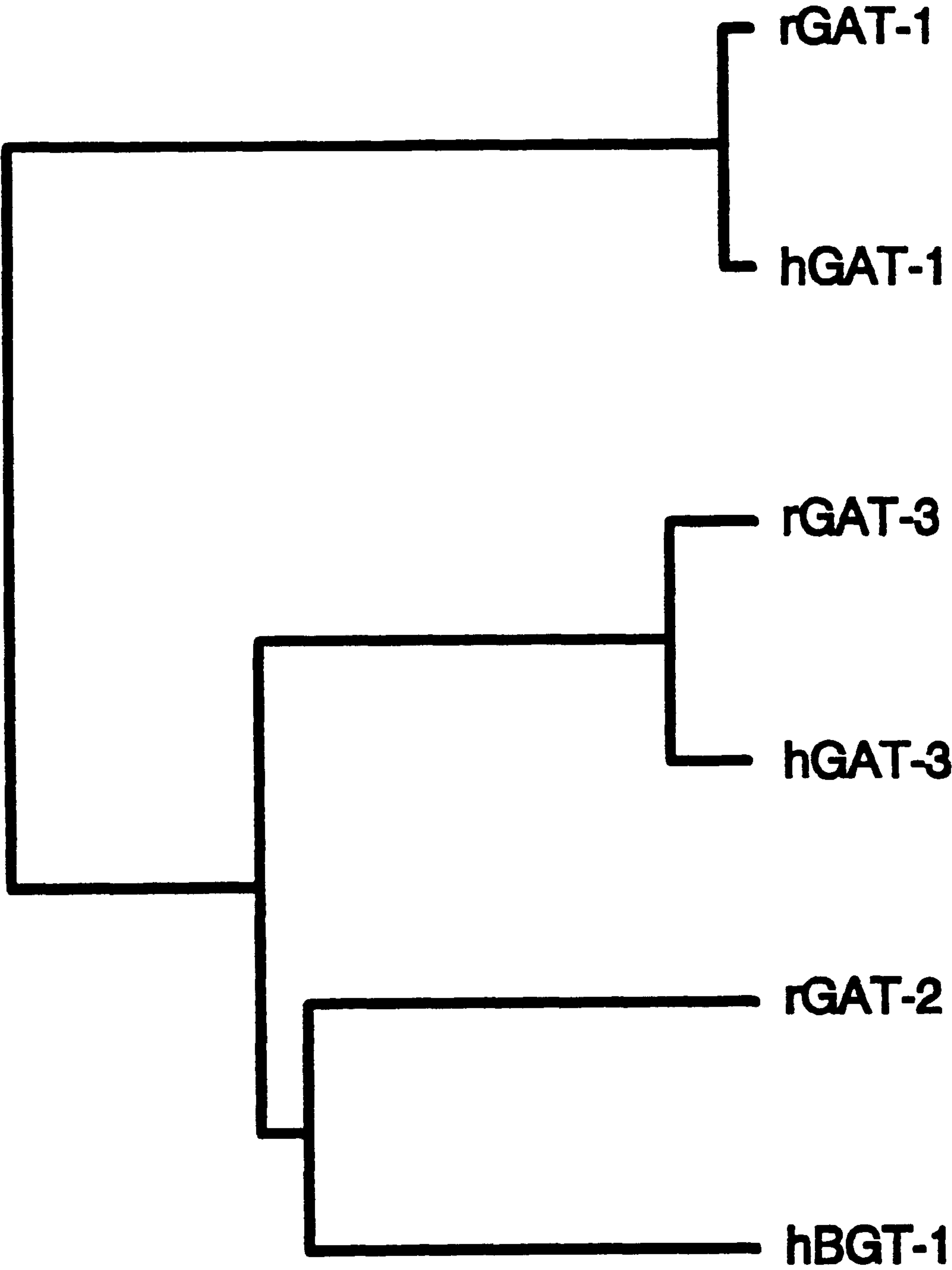


FIG. 17

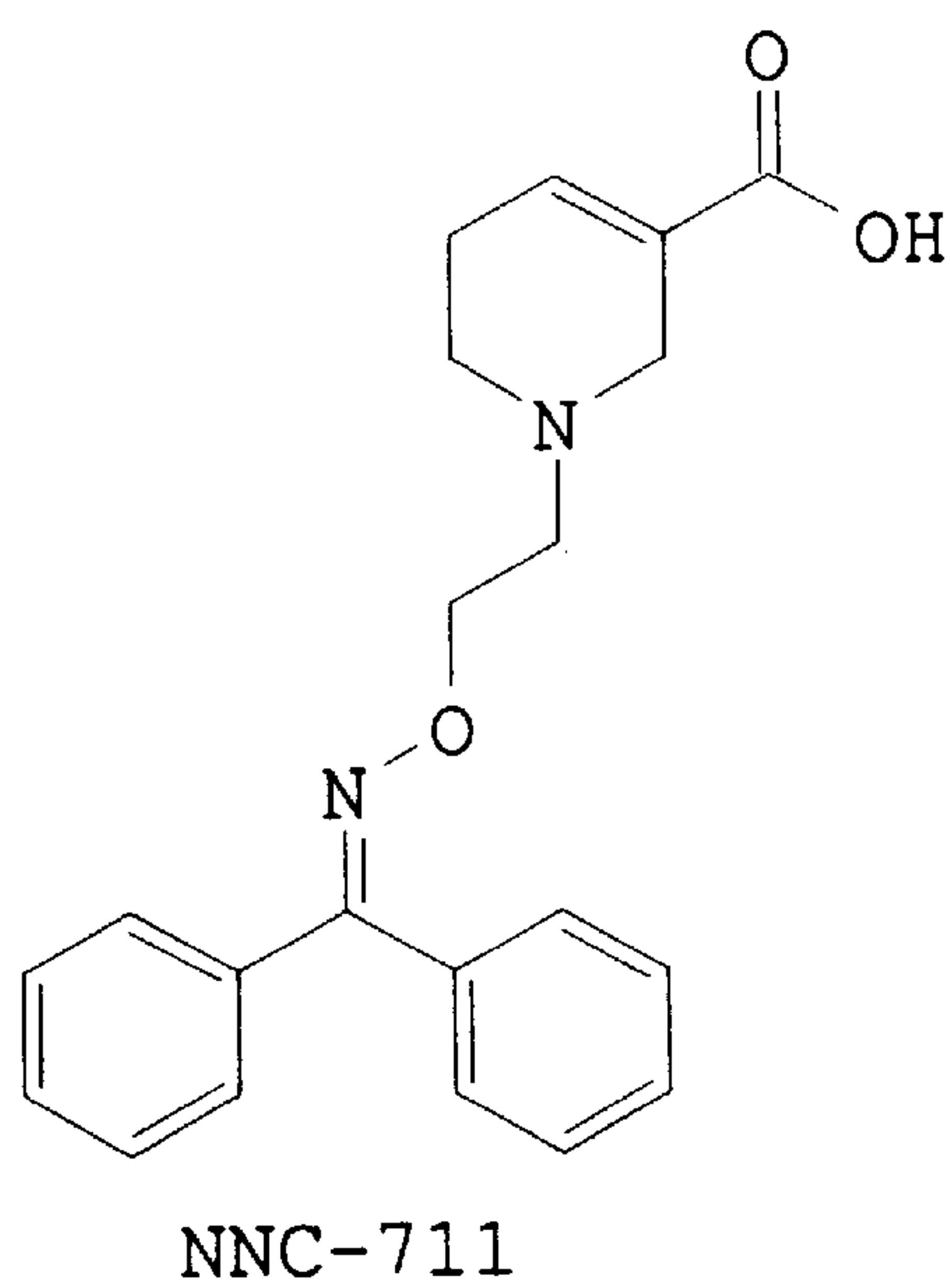
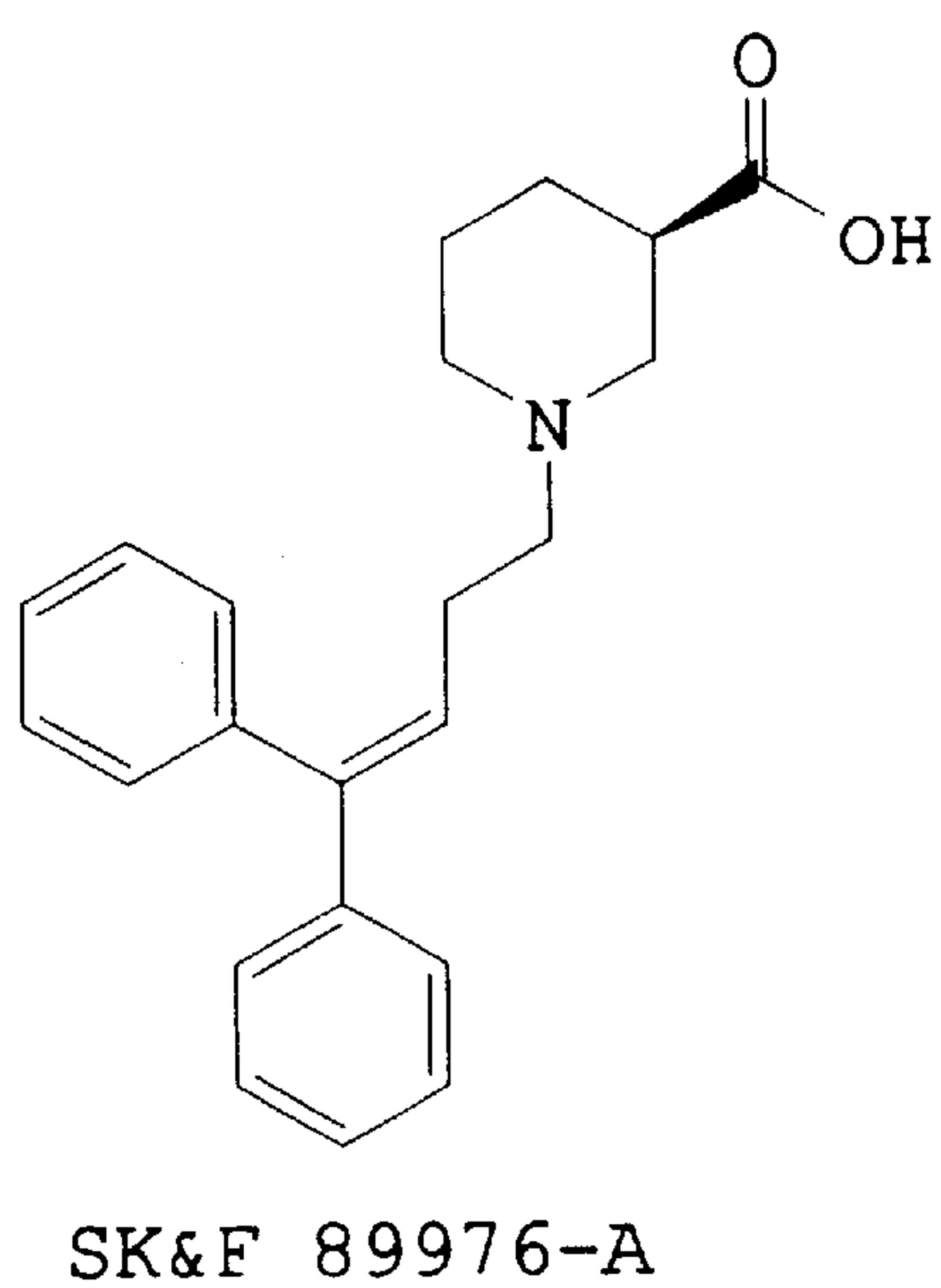
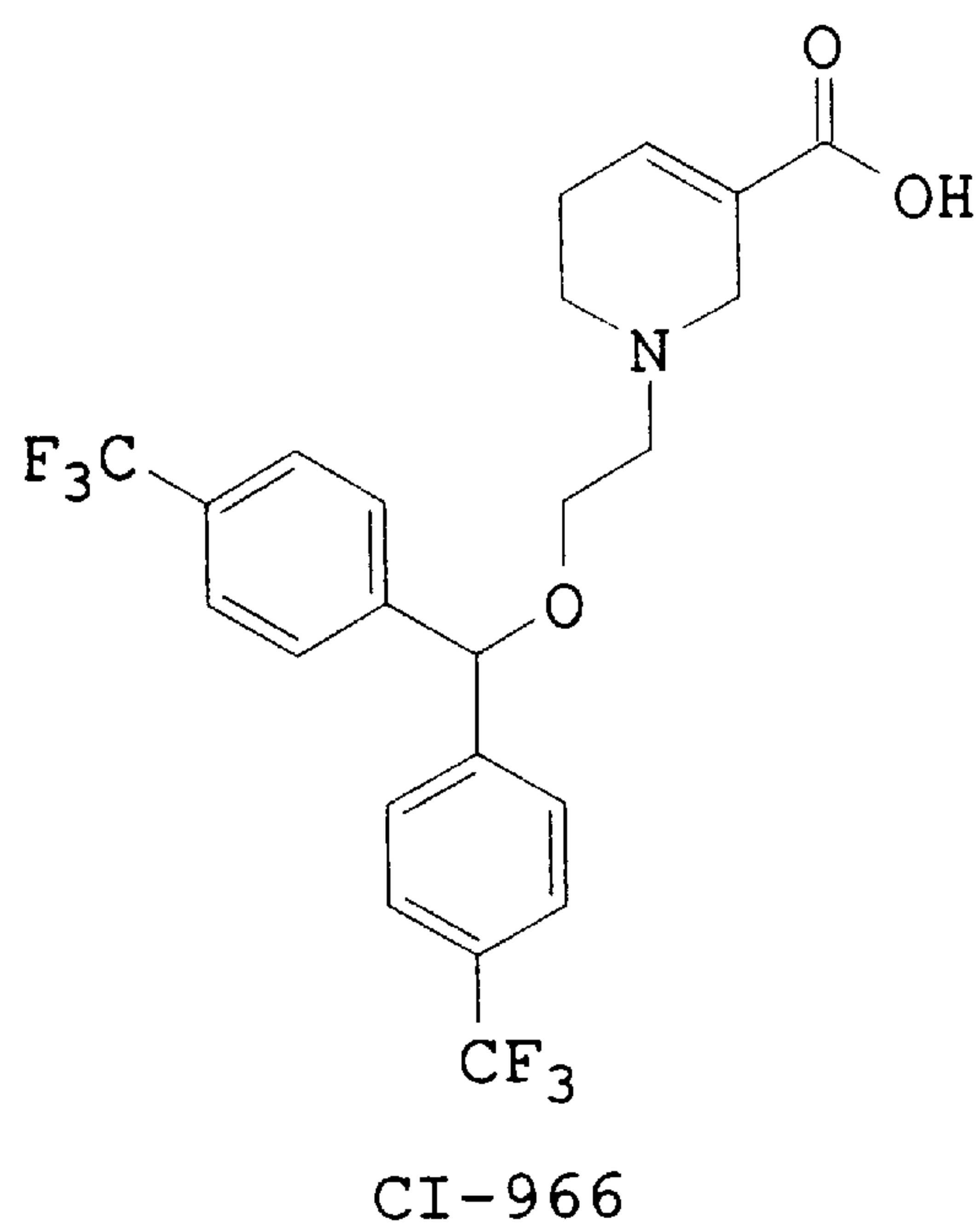
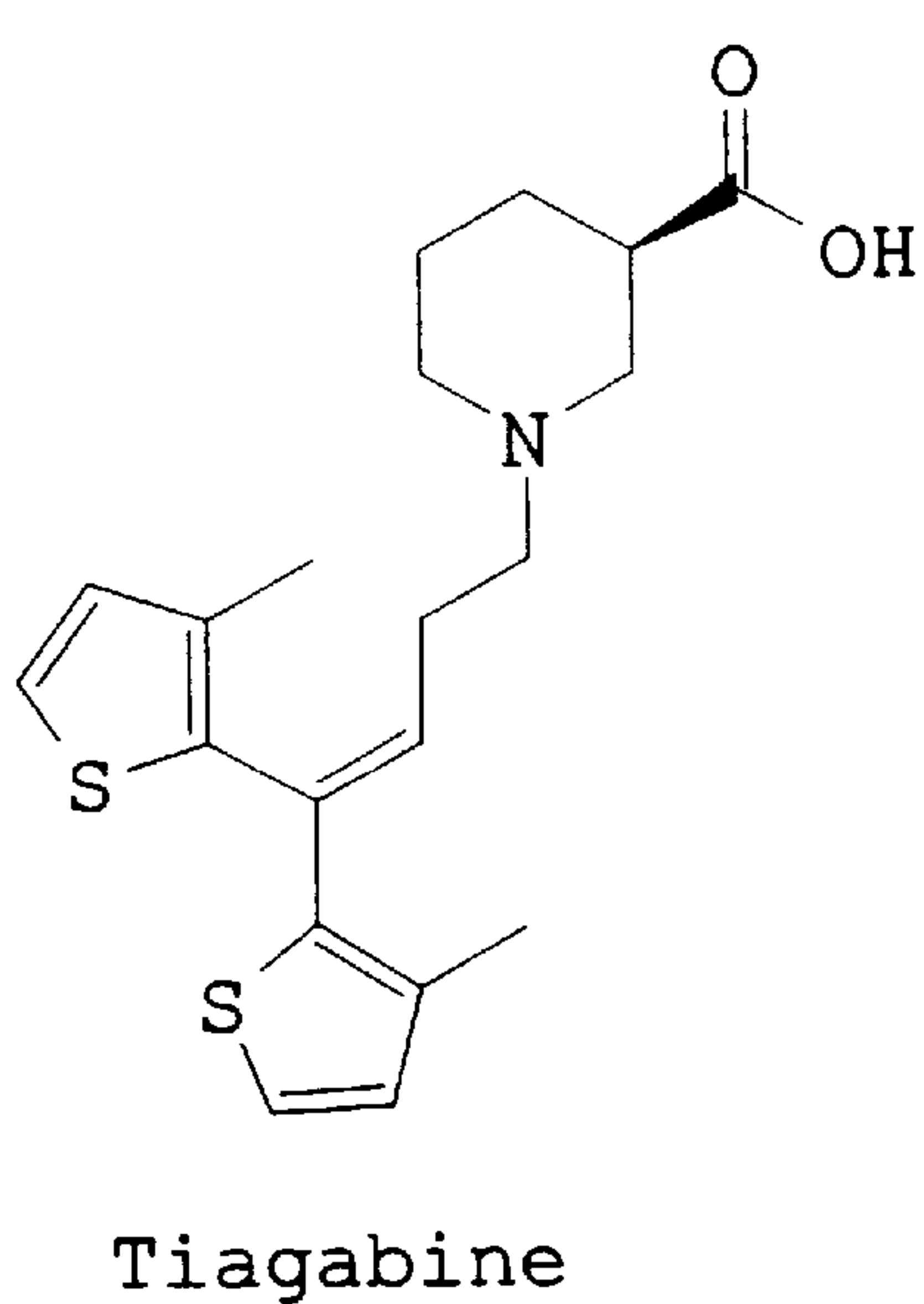
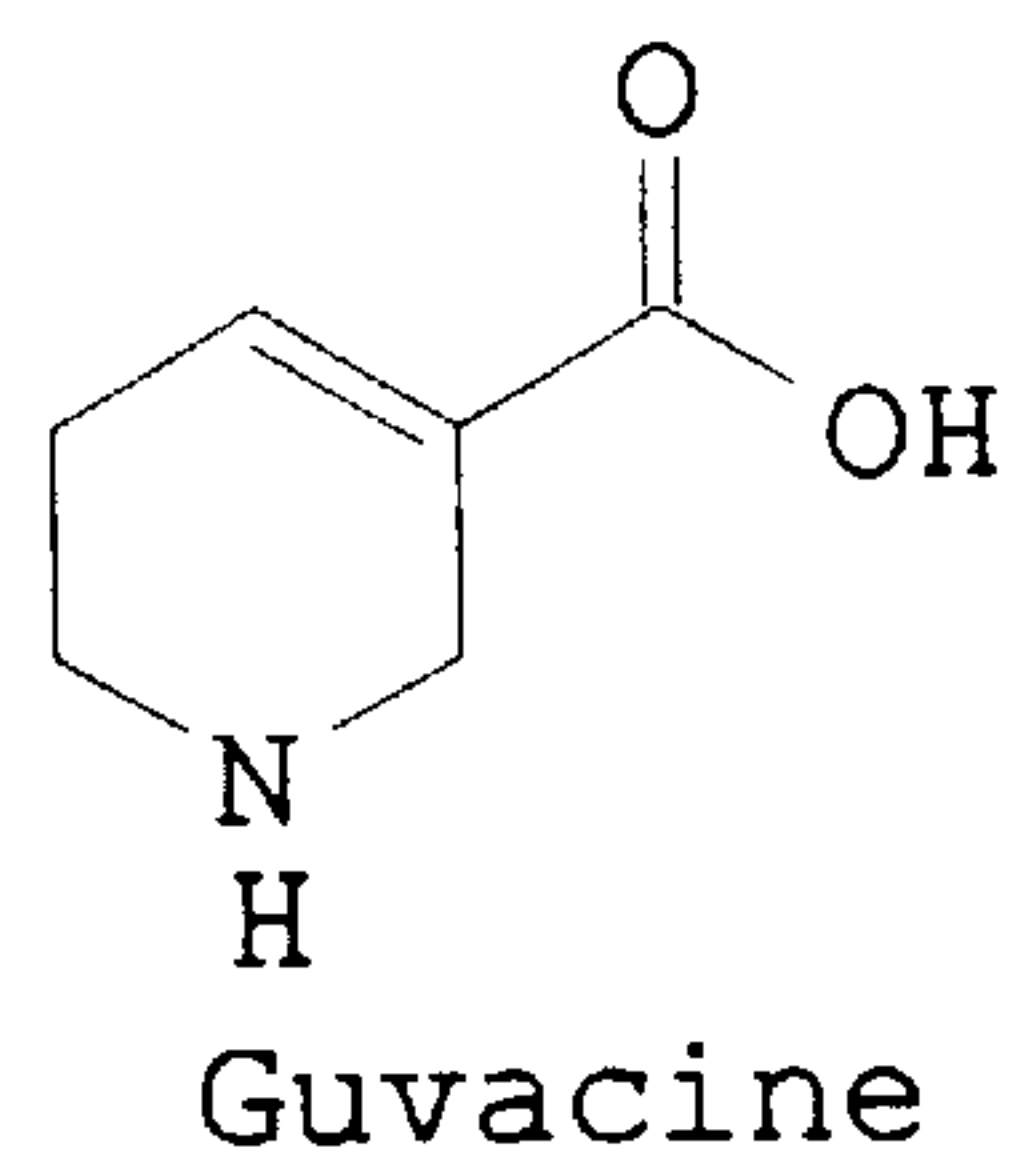
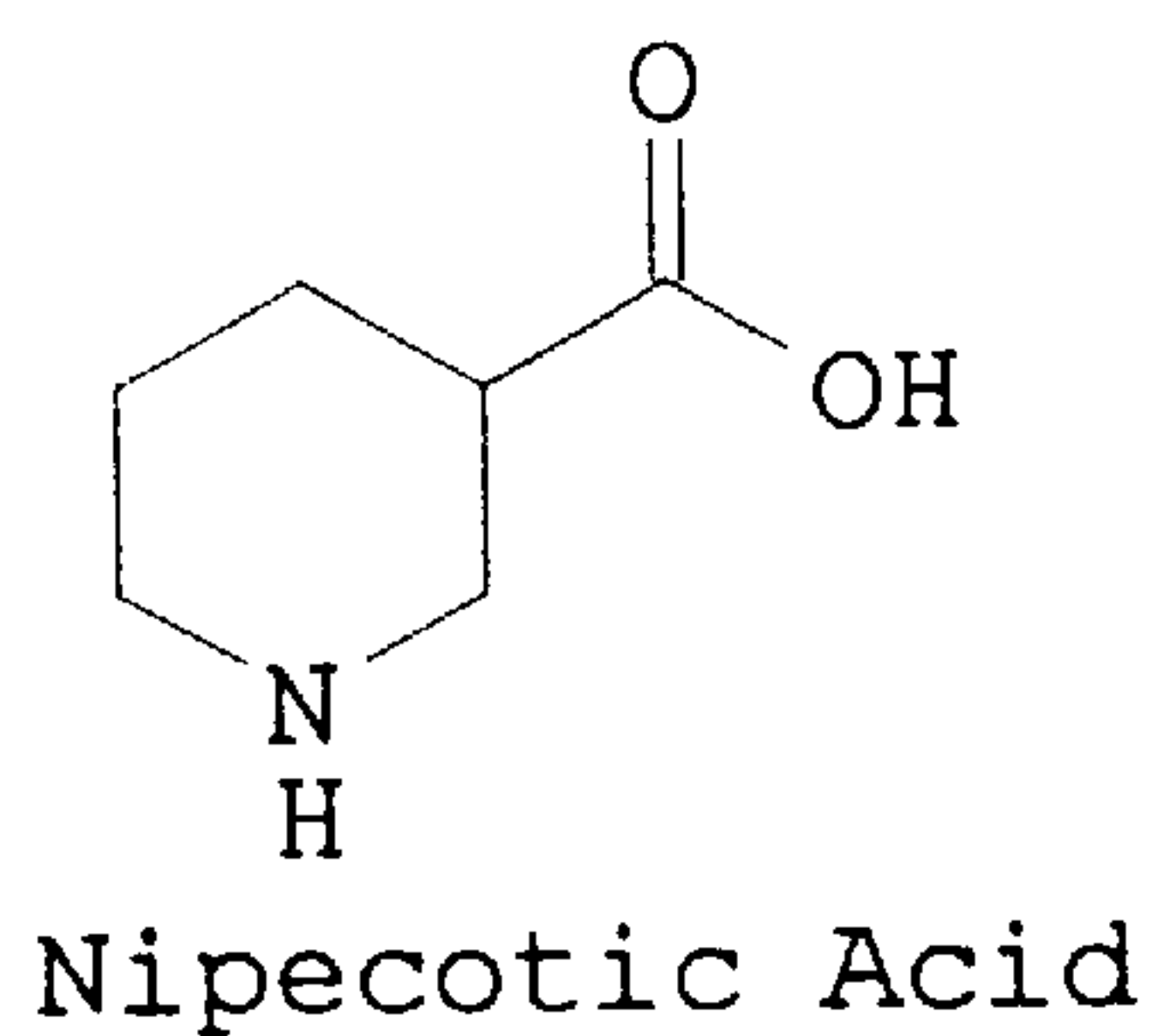


FIG. 18

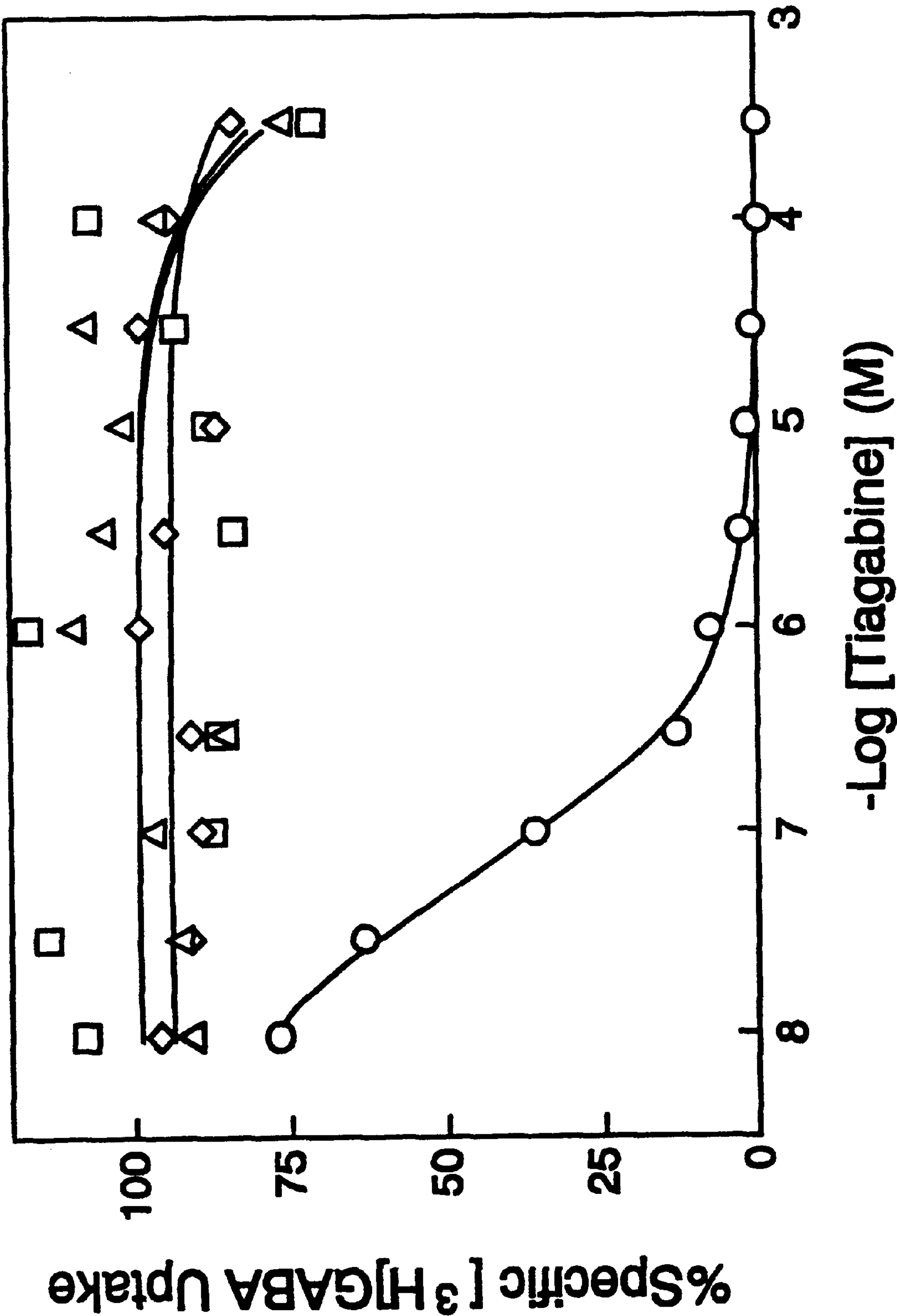


FIG. 19A



FIG. 19B



FIG. 19C

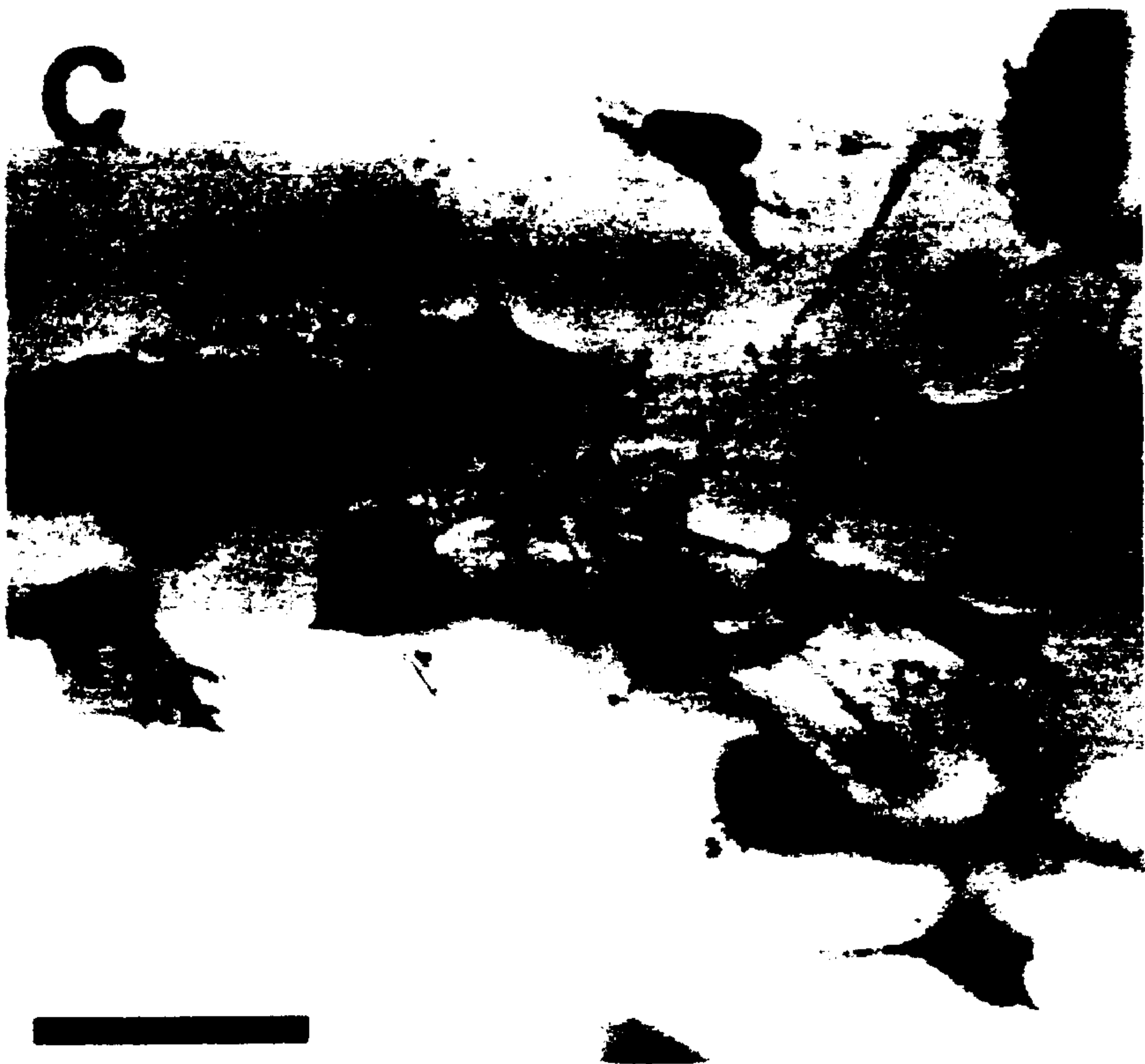


FIG. 20

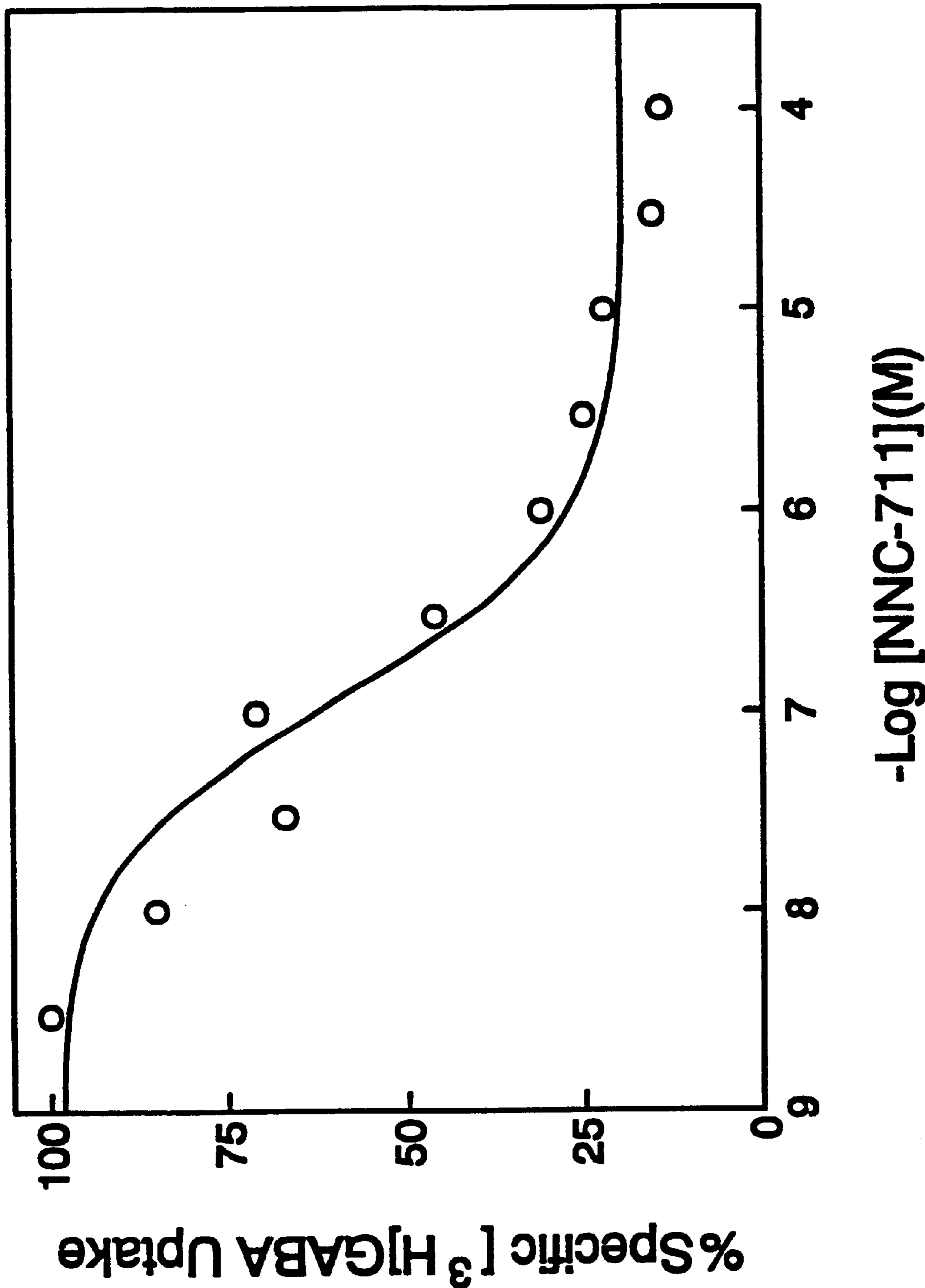


FIG. 21

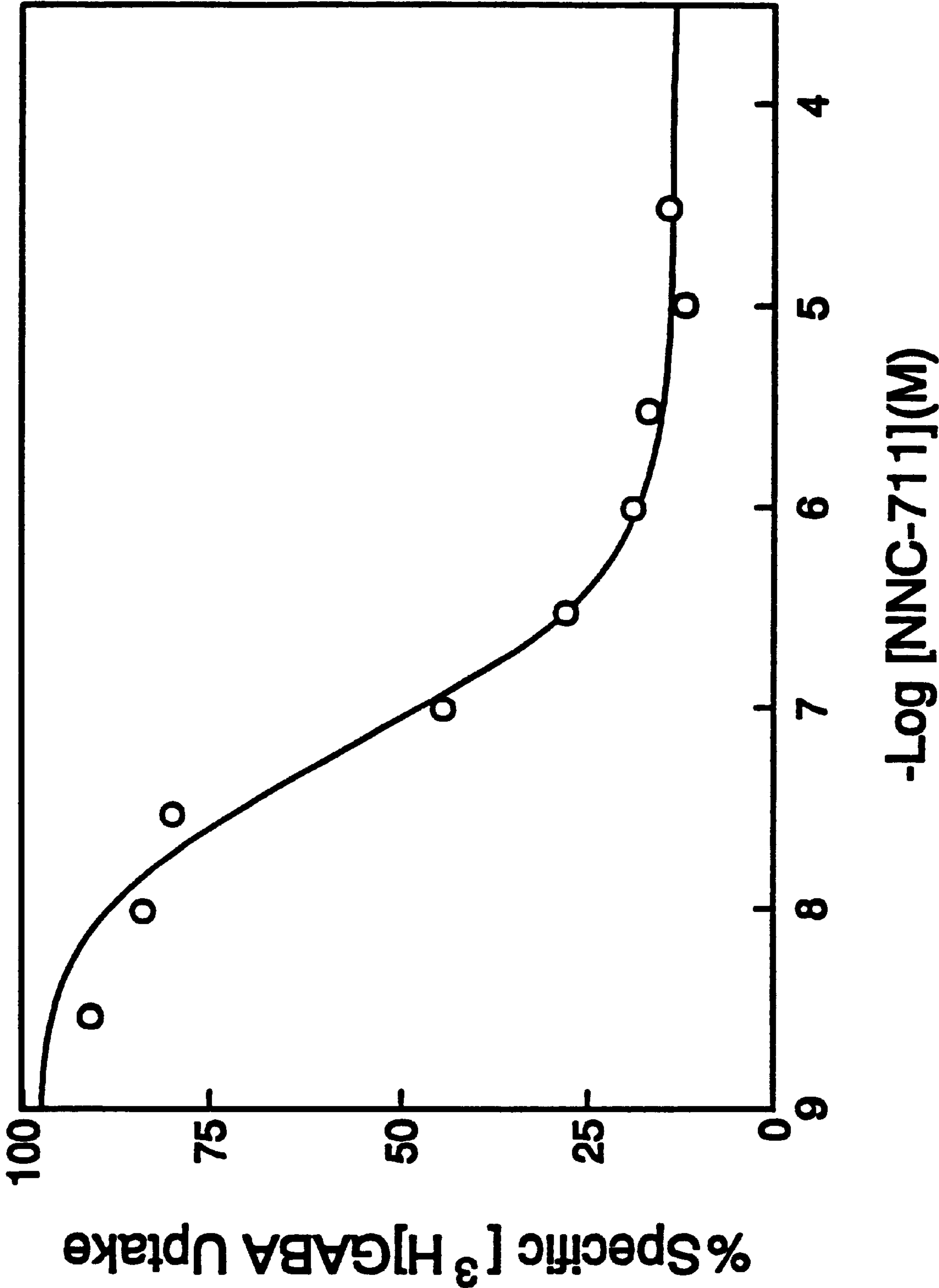
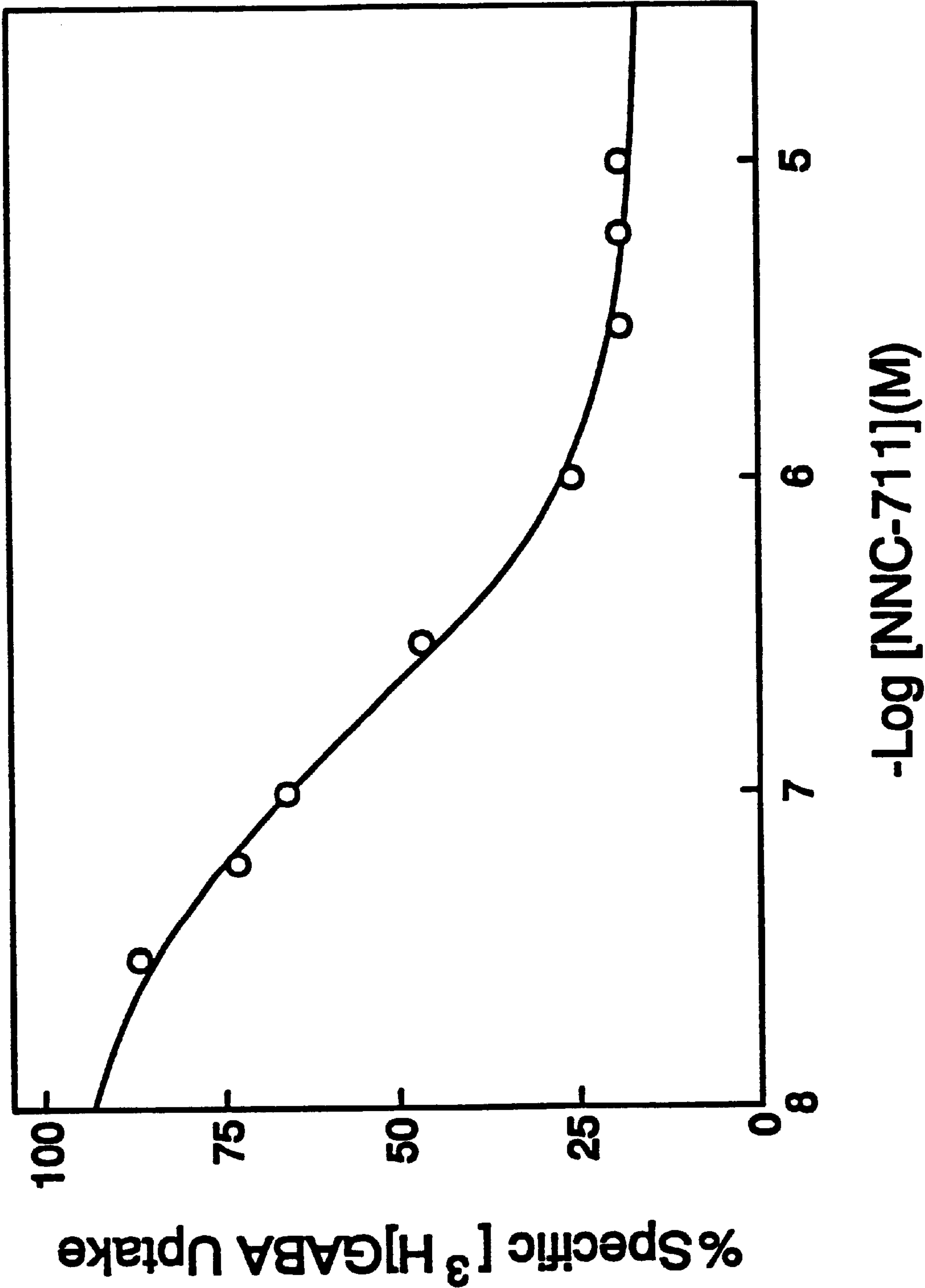


FIG. 22



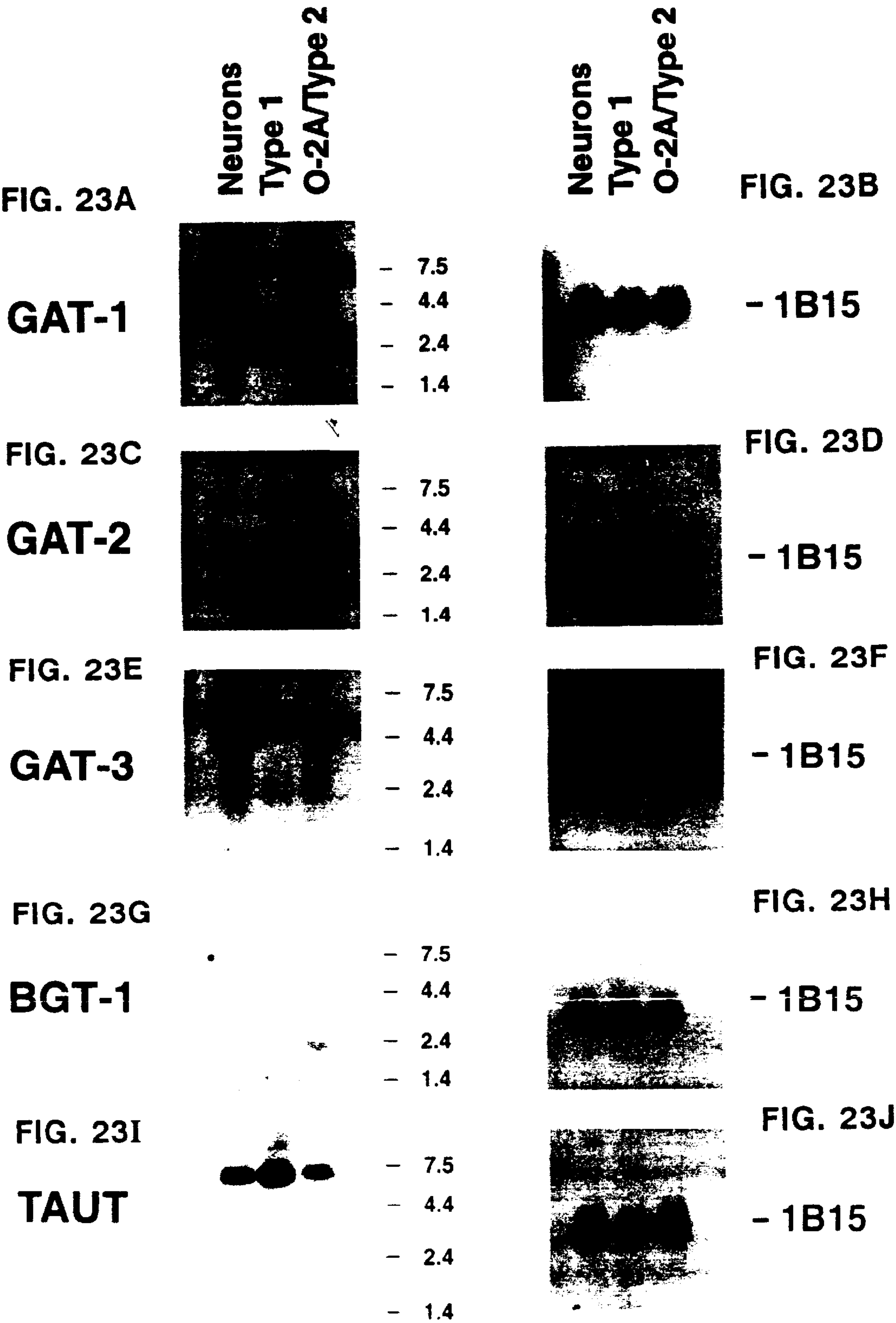


FIG. 24A



FIG. 24B

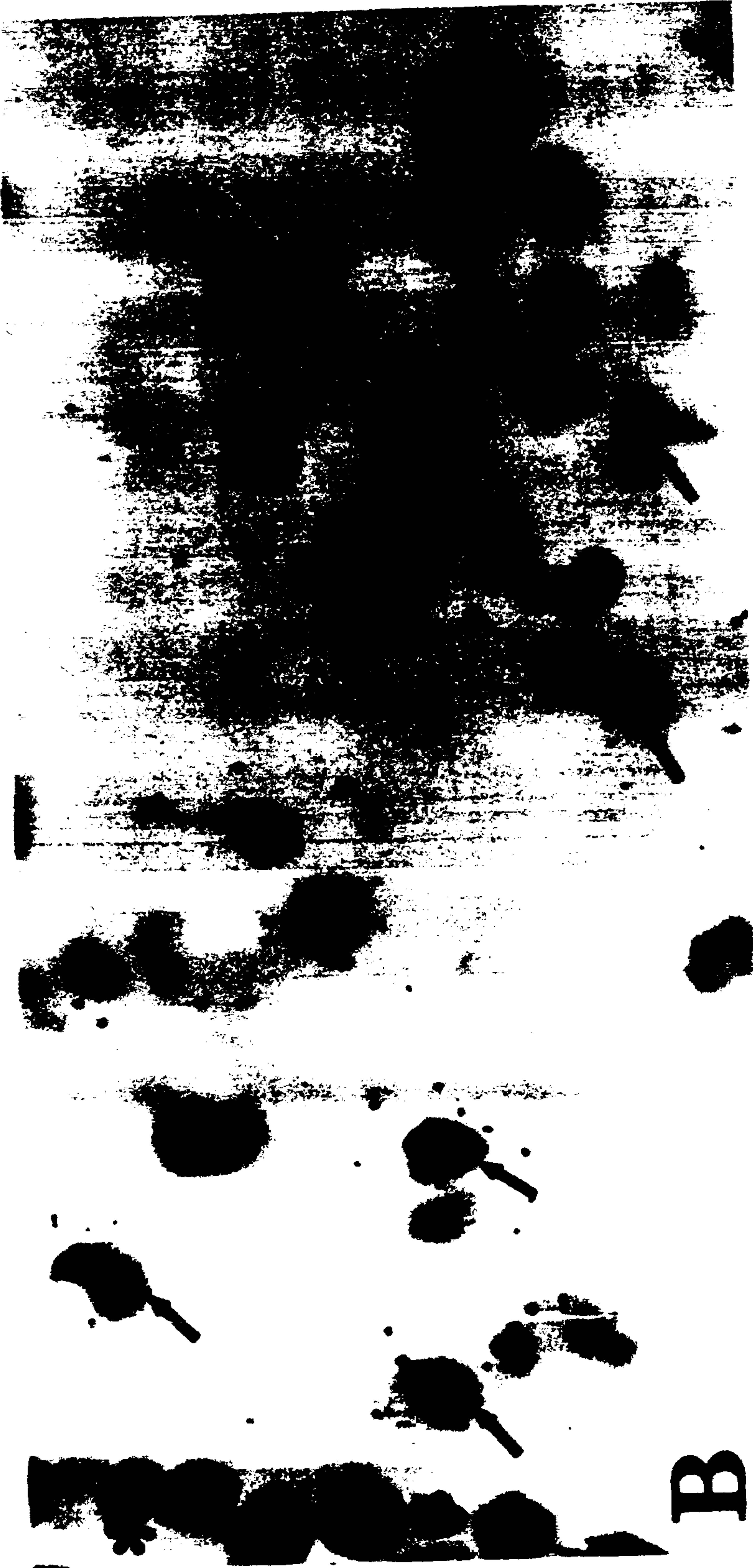


FIG. 24C



DNA ENCODING TAURINE AND GABA TRANSPORTERS AND USES THEREOF

This application is a continuation-in-part of U.S. Ser. No. 08/233,616, filed Apr. 25, 1994, now abandoned, which was a continuation-in-part of PCT International Application PCT/US93/01959, filed Mar. 4, 1993 designating the United States of America, which was a continuation-in-part of U.S. Ser. No. 07/959,936, filed Oct. 13, 1992, now abandoned, which was a continuation-in-part of U.S. Ser. No. 07/847,742, filed Mar. 4, 1992, now abandoned, the contents of all of which are hereby incorporated by reference into the subject application.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referred to by partial citations or by number within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Chemical neurotransmission is a multi-step process which involves release of neurotransmitter from the presynaptic terminal, diffusion across the synaptic cleft, and binding to receptors resulting in an alteration in the electrical properties of the postsynaptic neuron. For most neurotransmitters, transmission is terminated by the rapid uptake of neurotransmitter via specific, high-affinity transporters located in the presynaptic terminal and/or surrounding glial cells (39). Since inhibition of uptake by pharmacologic agents increases the levels of neurotransmitter in the synapse, and thus enhances synaptic transmission, neurotransmitter transporters provide important targets for therapeutic intervention.

The amino acid GABA is the major inhibitory neurotransmitter in the vertebrate central nervous system and is thought to serve as the neurotransmitter at approximately 40% of the synapses in the mammalian brain (22,37). GABAergic transmission is mediated by two classes of GABA receptors. The more prevalent is termed GABA_A, which is a multi-subunit protein containing an intrinsic ligand-gated chloride channel in addition to binding sites for a variety of neuroactive drugs including benzodiazepines and barbiturates (46,105). In contrast, GABA_B receptors couple to G-proteins and thereby activate potassium channels (5,46) and possibly alter levels of the second messenger cyclic AMP (46). Positive modulation of GABA_A receptors by diazepam, and related benzodiazepines has proven extremely useful in the treatment of generalized anxiety (116) and in certain forms of epilepsy (86).

Inhibition of GABA uptake provides a novel therapeutic approach to enhance inhibitory GABAergic transmission in the central nervous system (47,92). Considerable evidence indicates that GABA can be taken up by both neurons and glial cells, and that the transporters on the two cell types are pharmacologically distinct (24,47,92). A GABA transporter with neuronal-type pharmacology designated GAT-1 has previously been purified and cloned (31), but the molecular properties of other GABA transporters including glial transporter(s) have not yet been elucidated. We now report the cloning of two additional GABA transporters (GAT-2 and GAT-3) with distinct pharmacology and localization, revealing previously unsuspected heterogeneity in GABA transporters.

Termination of GABAergic neurotransmission is accomplished by uptake of neurotransmitter into the presynaptic terminal and the surrounding astroglial cells, which is mediated by high-affinity, sodium dependent transporters. Pharmacologic inhibition of uptake provides a novel mechanism for sustaining levels of neurotransmitter in the synapse and thereby increasing synaptic transmission.

Determining the efficacy of GABA transport inhibitors such as nipecotic acid in vivo has been hampered by their poor penetration of the blood-brain barrier, a property attributable to their high degree of hydrophilicity. In an effort to overcome this problem, Ali et al. (1985) examined the effect of adding lipophilic side chains to the nitrogen atom of various GABA transport blockers. Surprisingly, the addition of 4,4-diphenyl-3-butenyl side chains to nipecotic acid and guvacine (SK&F 89976-A and SK&F 100330-A, respectively) resulted in a 20-fold increase in potency when tested in brain synaptosomes. Since the original report, a number of other groups have synthesized similar derivatives such as CI-966 (Taylor et al., 1990) and Tiagabine (Nielsen et al., 1991). Importantly, these compounds all display anticonvulsive activity in laboratory animals (Yunger et al., 1984; Swinyard et al., 1991; Nielsen et al., 1991; Taylor et al., 1990; Suzdak et al., 1992).

Determining the site of action of the lipophilic GABA transport inhibitors is essential to understanding their mechanism of action, but is complicated by the heterogeneity of GABA transport. Early studies with cell culture systems suggested the existence of distinct neuronal and glial GABA transport systems (reviewed in Krogsgaard-Larsen et al., 1987). More recently, molecular cloning has identified four distinct GABA transporters termed GAT-1 (Guastella et al., 1990), GAT-2 and GAT-3 (disclosed herein), and BGT-1, the latter transporting both GABA and the osmolyte betaine (Yamauchi et al., 1992). A clone identical to GAT-3 was described by Clark et al. (1992) and termed GAT-B, and clones for all four GABA transporters have been identified in mice, though a different terminology was employed (Liu et al., 1993).

The specificities of known anticonvulsive agents were determined in order to examine the potency of four lipophilic transport inhibitors at each of the four cloned GABA transporters. We found that they all show a high degree of selectivity for GAT-1, indicating that their anticonvulsive activity is mediated via inhibition of GABA transport by this site.

In contrast to GAT-1, the relationship of GAT-2, GAT-3, and BGT-1 to the pharmacologically characterized neuronal and glial transport systems is not understood. While GAT-2 and GAT-3 display high affinity for β -alanine and low affinity for ACHC, suggesting a similarity to the glial transporter, their overall pharmacological profile indicates that they are distinct from this site. Additionally, the observation that GAT-3/GAT-B is located in neurons (Clark et al., 1992) indicates that neuronal transport is not limited to GAT-1, and that β -alanine sensitivity is not unique to glial GABA transport.

An additional level of complexity results from the heterogeneity of astrocytes. Two types of astrocytes, termed Type 1 and Type 2, have been described in cell cultures (for reviews see Raff, 1989; Miller et al., 1989). Interestingly, it has been suggested that Type 2 astrocytes and their precursor, termed O-2A, display a "neuronal-like" GABA transport pharmacology (Levi et al., 1983; Reynolds and Herschkowitz, 1984; Johnstone et al., 1986), suggesting that GAT-1 may not be restricted to neurons. A failure to recog-

nize and/or distinguish these cell types in many older studies complicates their interpretation.

Taurine (2-aminoethanesulfonic acid) is a sulfur containing amino acid present in high concentrations in mammalian brain as well as various non-neural tissues. Many functions have been ascribed to taurine in both the nervous system and peripheral tissues. The best understood (and phylogenetically oldest) function of taurine is as an osmoregulator (36,107). Osmoregulation is essential to normal brain function and may also play a critical role in various pathophysiological states such as epilepsy, migraine, and ischemia. The primary mechanism by which neurons and glial cells regulate osmolarity is via the selective accumulation and release of taurine. Taurine influx is mediated via specific, high-affinity transporters which may contribute to efflux as well. Since taurine is slowly degraded, transport is an important means of regulating extracellular taurine levels.

Taurine is structurally related to the inhibitory amino acid γ -aminobutyric acid (GABA) and exerts inhibitory effects on the brain, suggesting a role as a neurotransmitter or neuromodulator. Taurine can be released from both neurons and glial cells by receptor-mediated mechanisms as well as in response to cell volume changes (94). Its effects in the CNS may be mediated by GABA_A and GABA_B receptors (45,80) and by specific taurine receptors (109). Additionally, taurine can also regulate calcium homeostasis in excitable tissues such as the brain and heart (36,55), via an intracellular site of action. Together, the inhibitory and osmoregulatory properties of taurine suggest that it acts as a cytoprotective agent in the brain. Depletion of taurine results in retinal degeneration in cats (100), supporting a role in neuronal survival.

Although most animals possess the ability to synthesize taurine, many are unable to generate sufficient quantities and therefore rely on dietary sources. Taurine transport is thus critical to the maintenance of appropriate levels of taurine in the body. High affinity, sodium-dependent taurine uptake has been observed in brain and various peripheral tissues (36, 94), but little is known about the molecular properties of the taurine transporter(s). Cloning of the taurine transporter will not only help elucidate the function of this important neuroactive molecule, but may also provide important insight into novel therapeutic approaches to treat neurological disorders. cDNA clones (designated rB14b, K438-rB8b (the truncated clone is rB8b), and rB16a) encoding transporters for two novel GABA transporters and a taurine transporter, respectively, have been isolated from rat brain, and their functional properties have been examined in mammalian cells. The transporters encoded by rB14b and rB8b display high affinity for GABA ($K_m=4 \mu M$), and exhibit pharmacological properties distinct from the neuronal GABA transporter; the transporter encoded by rB16a displays high-affinity for taurine. All three are dependent on external sodium and chloride for transport activity. The nucleotide sequences of the three clones predict proteins of 602, 627, and 621 amino acids, respectively. Hydropathy analysis reveals stretches of hydrophobic amino acids suggestive of 12 transmembrane domains, similar to that proposed for other cloned neurotransmitter transporters. The cloning of two additional GABA transporters and a taurine transporter from rat brain reveals previously undescribed heterogeneity in inhibitory amino acid transporter genes.

The use of human gene products in the process of drug development offers significant advantages over those of other species, which may not exhibit the same pharmacological profiles. To facilitate this human-target based approach to drug design in the area of inhibitory amino acid

transporters, we used the nucleotide sequences of the rat GAT-2 and GAT-3 cDNAs to clone the human homologue of each gene. CDNA clones (designated hHE7a, hS3a, hFB16a, and hFB20a) encoding the human homologue of the two novel GABA transporters GAT-2 and GAT-3 have been isolated.

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding a mammalian GABA transporter. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid designated EVJB-rB14b (ATCC Accession No. 75203). In another embodiment of this invention, the nucleic acid molecule comprises a plasmid designated EVJB-rB8b (ATCC Accession No. 75201), containing the truncated rat GAT-3 nucleic acid.

This invention also provides an isolated nucleic acid molecule encoding a mammalian taurine transporter. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid designated EVJB-rB16a (ATCC Accession No. 75202).

This invention further provides isolated nucleic acid molecules encoding the human homologue of the mammalian GABA transporters. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid designated pcEXV-hGAT-3 (ATCC Accession No. 75324). In another embodiment of this invention, the nucleic acid molecule comprises a plasmid designated pBluescript hHE7a (ATCC Accession No. 75322). In another embodiment of this invention, the nucleic acid molecule comprises the plasmid pBluescript-hS3a (ATCC Accession No. 75323).

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a mammalian GABA transporter. This invention also provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a mammalian taurine transporter. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human GABA transporter. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human taurine transporter.

This invention further provides an antisense oligonucleotide having a sequence capable of binding specifically to an mRNA molecule encoding a mammalian GABA transporter so as to prevent translation of the mRNA molecule. This invention also provides an antisense oligonucleotide having a sequence capable of binding specifically to an mRNA molecule encoding a mammalian taurine transporter so as to prevent translation of the mRNA molecule. This invention also provides an antisense oligonucleotide having a sequence capable of binding specifically to an mRNA molecule encoding a human GABA transporter so as to prevent translation of the mRNA molecule. This invention also provides an antisense oligonucleotide having a sequence capable of binding specifically to an mRNA molecule encoding a human taurine transporter so as to prevent translation of the mRNA molecule.

A monoclonal antibody directed to a mammalian GABA transporter is provided by this invention. A monoclonal

antibody directed to a mammalian taurine transporter is also provided by this invention. A monoclonal antibody directed to a human GABA transporter is also provided by this invention. A monoclonal antibody directed to a human taurine transporter is also provided by this invention.

This invention provides a pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a mammalian GABA transporter and a pharmaceutically acceptable carrier as well as a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of GABA transporter and a pharmaceutically acceptable carrier.

A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a mammalian taurine transporter and a pharmaceutically acceptable carrier as well as a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a taurine transporter and a pharmaceutically acceptable carrier is also provided by this invention.

A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human GABA transporter and a pharmaceutically acceptable carrier as well as a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human GABA transporter and a pharmaceutically acceptable carrier is also provided by this invention.

A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human taurine transporter and a pharmaceutically acceptable carrier as well as a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human taurine transporter and a pharmaceutically acceptable carrier is also provided by this invention.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a mammalian GABA transporter so positioned within such genome as to be transcribed into antisense mRNA complementary to mRNA encoding the GABA transporter and when hybridized to mRNA encoding the GABA transporter, the complementary mRNA reduces the translation of the mRNA encoding the GABA transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a mammalian taurine transporter so positioned within such genome as to be transcribed into antisense mRNA complementary to mRNA encoding the taurine transporter and when hybridized to mRNA encoding the taurine transporter, the complementary mRNA reduces the translation of the mRNA encoding the taurine transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a human GABA transporter so positioned within such genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the human GABA transporter and when hybridized to mRNA encoding the human GABA transporter, the antisense mRNA thereby reduces the translation of mRNA encoding the human GABA transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a human taurine transporter so positioned within such genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the human taurine transporter and

when hybridized to mRNA encoding the human taurine transporter, the antisense mRNA thereby reduces the translation of mRNA encoding the human taurine transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a mammalian GABA transporter so positioned within such genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the transporter and when hybridized to mRNA encoding the transporter, the antisense mRNA thereby prevents the translation of mRNA encoding the transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a mammalian taurine transporter so positioned within such genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the transporter and when hybridized to mRNA encoding the transporter, the antisense mRNA thereby prevents the translation of mRNA encoding the transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a human GABA transporter so positioned within such genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the transporter and when hybridized to mRNA encoding the human GABA transporter, the antisense mRNA thereby prevents the translation of mRNA encoding the human GABA transporter.

This invention also provides a transgenic, nonhuman mammal whose genome comprises DNA encoding a human taurine transporter so positioned within such genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the human taurine transporter and when hybridized to mRNA encoding the human taurine transporter, the antisense mRNA thereby prevents the translation of mRNA encoding the human taurine transporter.

This invention provides a method of screening drugs to identify drugs which specifically interact with, and bind to, a mammalian GABA transporter on the surface of a cell which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding a mammalian GABA transporter, the protein encoded thereby is expressed on the cell surface, with a plurality of drugs, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, a mammalian GABA transporter.

This invention provides a method of screening drugs to identify drugs which specifically interact with, and bind to, a mammalian taurine transporter on the surface of a cell which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding a mammalian taurine transporter, the protein encoded thereby is expressed on the cell surface, with a plurality of drugs, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, a mammalian taurine transporter.

This invention provides a method of screening drugs to identify drugs which specifically interact with, and bind to, a human GABA transporter on the surface of a cell which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding a human GABA transporter, the protein encoded thereby is expressed on the cell surface, with a plurality of drugs, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, a human GABA transporter.

This invention provides a method of screening drugs to identify drugs which specifically interact with, and bind to,

a human taurine transporter on the surface of a cell which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding a human taurine transporter, the protein encoded thereby is expressed on the cell surface, with a plurality of drugs, determining those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, a human taurine transporter.

This invention also provides a method of determining the physiological effects of expressing varying levels of mammalian GABA transporters which comprises producing a transgenic nonhuman animal whose levels of mammalian GABA transporter expression are varied by use of an inducible promoter which regulates mammalian GABA transporter expression.

This invention also provides a method of determining the physiological effects of expressing varying levels of mammalian taurine transporters which comprises producing a transgenic nonhuman animal whose levels of mammalian taurine transporter expression are varied by use of an inducible promoter which regulates mammalian taurine transporter expression.

This invention also provides a method of determining the physiological effects of expressing varying levels of human GABA transporters which comprises producing a transgenic nonhuman animal whose levels of human GABA transporter expression are varied by use of an inducible promoter which regulates human GABA transporter expression.

This invention also provides a method of determining the physiological effects of expressing varying levels of human taurine transporters which comprises producing a transgenic nonhuman animal whose levels of human taurine transporter expression are varied by use of an inducible promoter which regulates human taurine transporter expression.

This invention further provides a method of determining the physiological effects of expressing varying levels of mammalian GABA transporters which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of mammalian GABA transporter.

This invention further provides a method of determining the physiological effects of expressing varying levels of mammalian taurine transporters which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of mammalian taurine transporter.

This invention further provides a method of determining the physiological effects of expressing varying levels of human GABA transporters which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of human GABA transporter.

This invention further provides a method of determining the physiological effects of expressing varying levels of human taurine transporters which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of human taurine transporter.

This invention provides a method for diagnosing a predisposition to a disorder associated with the expression of a specific mammalian GABA transporter allele and a method for diagnosing a predisposition to a disorder associated with the expression of a specific mammalian taurine transporter allele which comprises: a.) obtaining DNA of subjects suffering from the disorder; b.) performing a restriction digest of the DNA with a panel of restriction enzymes; c.) electrophoretically separating the resulting DNA fragments on a sizing gel; d.) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to DNA encoding a mammalian GABA or a mammalian taurine

transporter and labeled with a detectable marker; e.) detecting labeled bands which have hybridized to the DNA encoding a mammalian GABA or taurine transporter labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; f.) preparing DNA obtained for diagnosis by steps a-e; and g.) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step e and the DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

This invention provides a method for diagnosing a predisposition to a disorder associated with the expression of a specific human GABA transporter allele or a specific human taurine transporter allele which comprises: a.) obtaining DNA of subjects suffering from the disorder; b.) performing a restriction digest of the DNA with a panel of restriction enzymes; c.) electrophoretically separating the resulting DNA fragments on a sizing gel; d.) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to DNA encoding a human GABA or human taurine transporter and labeled with a detectable marker; e.) detecting labeled bands which have hybridized to the DNA encoding a human GABA or human taurine transporter labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; f.) preparing DNA obtained for diagnosis by steps a-e; and g.) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step e and the DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

This invention provides a method for determining whether a substrate not known to be capable of binding to a mammalian transporter can bind to the mammalian GABA transporter which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding the GABA transporter with the substrate under conditions permitting binding of substrates known to bind to a transporter, detecting the presence of any of the substrate bound to the GABA transporter, and thereby determining whether the substrate binds to the GABA transporter.

This invention provides a method for determining whether a substrate not known to be capable of binding to a taurine transporter can bind to a taurine transporter which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding the taurine transporter with the substrate under conditions permitting binding of substrates known to bind to a transporter, detecting the presence of any of the substrate bound to the taurine transporter, and thereby determining whether the substrate binds to the taurine transporter.

This invention provides a method for determining whether a substrate not known to be capable of binding to a human GABA transporter can bind to a human GABA transporter which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding the human GABA transporter with the substrate under conditions permitting binding of substrates known to bind to a transporter, detecting the presence of any of the substrate bound to the human GABA transporter, and thereby determining whether the substrate binds to the human GABA transporter.

This invention provides a method for determining whether a substrate not known to be capable of binding to a

human taurine transporter can bind to a human taurine transporter which comprises contacting a mammalian cell comprising an isolated DNA molecule encoding the human taurine transporter with the substrate under conditions permitting binding of substrates known to bind to a transporter, detecting the presence of any of the substrate bound to the human taurine transporter, and thereby determining whether the substrate binds to the human taurine transporter.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A–1D. Nucleotide Sequence and Deduced Amino Acid Sequence of the Mammalian GABA transporter encoded by GAT-2 (rB14b) (Seq. I.D. Nos. 1 and 2). Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the putative initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown.

FIGS. 2A–2D. Nucleotide Sequence and Deduced Amino Acid Sequence of the Mammalian GABA transporter encoded by GAT-3 (K438-rB8b) (Seq. I.D. Nos. 3 and 4). Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the putative initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown.

FIGS. 3A–3D. Nucleotide Sequence and Deduced Amino Acid Sequence of the Taurine transporter encoded by rB16a (Seq. I.D. Nos. 5 and 6). Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the putative initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown.

FIGS. 4A–4B. Deduced amino acid sequence and putative membrane topology of GABA transporter GAT-2 (rB14b). Deduced amino acid sequence by translation of a long open reading frame in rB14b is shown. Residues which are identical to those of GAT-3 (K438-rB8b) are shaded. Membrane topology is modeled after that proposed for GAT-1 (21).

FIGS. 5A–5B. Deduced amino acid sequence and putative membrane topology of taurine transporter (rB16a). Deduced amino acid sequence by translation of a long open reading frame in rB16a is shown. Membrane topology is modeled after that proposed for GAT-1 (21).

FIGS. 6A–6D. Alignment of the novel GABA transporters with the rat neuronal GABA transporter, the betaine transporter, and the glycine transporter. The twelve putative α -helical membrane spanning domains (I–XII) are bracketed. Residues identical to those of GAT-2 are shaded. GAT-2 is the GABA transporter encoded by rB14b; GAT-3 is the GABA transporter encoded by K438-rB8b; GAT-1 is the rat neuronal GABA transporter (21) (Seq. I.D. No. 11), Betaine is the dog betaine transporter (79) (Seq. I.D. No. 12), and Glycine is the rat glycine transporter (68) (Seq. I.D. No. 13).

FIG. 7A. GABA transport by COS cells transfected with clone rB14b. Non-transfected COS cells (control) or COS cells transfected with GAT-2 were incubated for 10 minutes (37° C.) with 50 nM [^{3} H]GABA in either HBS (150 mM NaCl) or in a similar solution in which Na⁺ was replaced by equimolar Li⁺ (Na⁺-free), or Cl⁻ was replaced by acetate (in some experiments, calcium gluconate was used instead of calcium acetate; Cl⁻-free). Data show the specific uptake of GABA, expressed as pmoles/mg protein cellular protein. Data are from a single experiment that was repeated with similar results.

FIG. 7B. GABA transport by COS cells transfected with either clone rB8b or clone K438-rB8b. Non-transfected COS cells (control) or COS cells transfected with GAT-3 were incubated for 10 minutes (37° C.) with 50 nM [^{3} H]GABA in either HBS (150 mM NaCl) or in a similar solution in which Na⁺ was replaced by equimolar Li⁺ (Na⁺-free), or Cl⁻ was replaced by acetate (in some experiments, calcium gluconate was used instead of calcium acetate; Cl⁻-free). Data show the specific uptake of GABA, expressed as pmoles/mg protein cellular protein. Data are from a single experiment that was repeated with similar results.

FIG. 8A. Concentration dependence of GABA transport. COS cells transfected with GAT-2 were incubated with the indicated concentrations of [^{3} H]GABA for 30 seconds and the accumulated radioactivity was determined. The specific activity of the [^{3} H]GABA was reduced with unlabeled GABA. Data represent specific transport expressed as nmoles per minute per mg protein, and are from a single experiment that was repeated with similar results (see Text).

FIG. 8B. Concentration dependence of GABA transport. COS cells transfected with GAT-3 were incubated with the indicated concentrations of [^{3} H]GABA for 30 seconds and the accumulated radioactivity was determined. The specific activity of the [^{3} H]GABA was reduced with unlabeled GABA. Data represent specific transport expressed as nmoles per minute per mg protein, and are from a single experiment that was repeated with similar results (see Text).

FIG. 9A. Localization of GABA transporters. Northern blot analysis of mRNAs encoding GAT-2 (rB14b) and GAT-3 (rB8b or K438-rB8b). Total RNA (25 μ g) from rat brain and liver was separated by formaldehyde/agarose gel electrophoresis, blotted to nylon membranes, and hybridized at high stringency with ^{32} P-labeled GABA transporter cDNAs (rB14b and rB8b or K438-rB8b). The autoradiogram was developed after a four day exposure. The locations of ribosomal RNAs are indicated at the side. The hybridizing transcripts are $\approx$ 2.4 kb (GAT-2) and $\approx$ 4.7 kb (GAT-3).

FIG. 9B. Localization of GABA transporters. Tissue distribution of mRNAs encoding GAT-1, GAT-2, and GAT-3 as determined by PCR. Single-stranded cDNA converted from poly A⁺ RNA was used for PCR amplification (30 cycles) of GABA transporter cDNA sequences. Amplified products were detected by hybridization with specific oligonucleotide probes; autoradiograms of the Southern blots are shown. GAT-1 is the neuronal GABA transporter. GAT-2 is the transporter encoded by rB14b. GAT-3 is the transporter encoded by K438-rB8b. Equivalent samples of poly A⁺ RNA (not treated with reverse transcriptase) subjected to identical PCR conditions showed no hybridization with the three probes (not shown). Cyclophilin cDNA was amplified to an equal extent from all tissues examined (not shown). Each experiment was repeated at least once with similar results.

FIGS. 10A–10C. Alignment of the taurine transporter with the GABA transporter GAT-1, the betaine transporter, and the glycine transporter. The twelve putative α -helical membrane spanning domains (I–XII) are bracketed. Residues identical to those of the taurine transporter are shaded. Taurine is the taurine transporter encoded by rB16a (Seq. I.D. No. 6); GAT-1 is the rat brain GABA transporter (21) (Seq. I.D. No. 11); Betaine is the dog betaine transporter (79) (Seq. I.D. No. 12); Glycine is the rat glycine transporter (68) (Seq. I.D. No. 13).

FIG. 11. Taurine transport by COS cells transfected with clone rB16a. Non-transfected COS cells (control) or COS cells transfected with rB16a were incubated for 10 minutes

(37° C.) with 50 nM [^3H]taurine in either HBS (150 mM NaCl) or in a similar solution in which Na^+ was replaced by equimolar Li^+ (Na^+ -free), or Cl^- was replaced by acetate (Cl^- -free). Data show the specific uptake of taurine, expressed as % of control cells. Each bar represents the mean $\pm$ SEM of 3–7 experiments.

FIG. 12. Concentration dependence of taurine transport. COS cells transfected with rB16a were incubated with the indicated concentrations of [^3H]taurine for 30 seconds and the accumulated radioactivity was determined. The specific activity of [^3H]taurine was reduced with unlabeled taurine. Data represent specific transport expressed as nmoles per minute per mg protein, and are from a single experiment that was repeated with similar results (see Text).

FIG. 13A. Localization of the taurine transporter. Tissue distribution of mRNA encoding the taurine transporter as determined by PCR. Single-stranded cDNA converted from poly A+ RNA was used for PCR amplification (30 cycles) of taurine transporter cDNA from a variety of rat tissues. A plasmid containing the cloned taurine transporter was amplified under identical conditions as a control. Amplified products were detected by hybridization with an oligonucleotide probe specific to the taurine transporter; an autoradiogram of the Southern blot is shown. Equivalent samples of poly A+ RNA (not treated with reverse transcriptase) subjected to identical PCR conditions showed no hybridization with the transporter probe (not shown), indicating that the signals obtained with cDNA were not a result of genomic DNA contamination. The experiment was repeated with similar results.

FIG. 13B. Localization of the taurine transporter. Northern blot analysis of mRNA encoding the taurine transporter. Poly A+ RNA (5 μg) from a variety of rat tissues was separated by formaldehyde/agarose gel electrophoresis, blotted to a nylon membrane, and hybridized at high stringency with ^{32}P -labeled taurine transporter cDNA (rB16a). The autoradiogram was developed after an overnight exposure. Size standards are indicated at the left in kilobases. The hybridizing transcript is ~ 6.2 kb.

FIGS. 14A–14B. Nucleotide Sequence and Deduced Amino Acid of Human Transporters. Sequence of the Human GAT-2 GABA Transporter (Seq. I.D. Nos. 7 and 8). Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the first nucleotide in a partial cDNA clone. Deduced amino acid sequence by translation of a long open reading frame is shown.

FIGS. 15A–15D. Nucleotide Sequence and Deduced Amino Acid of Human Transporters. Sequence of the Human GAT-3 GABA Transporter (Seq. I.D. Nos. 9 and 10). Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the putative initiating methionine and ending in the terminating codon. Deduced amino acid sequence by translation of a long open reading frame is shown.

FIG. 16. Amino acid relationships of cloned GABA transporters. The amino acid sequences of rat GAT-1 (Guastella et al., 1990), human GAT-1 (Nelson et al., 1990), rat GAT-2 (Borden et al., 1992), rat GAT-3 (Borden et al., 1992), human GAT-3 (Borden et al., 1994), and human BGT-1 (Borden et al., 1994) were compared using the pileup program (Genetics Computer Group, Inc., Madison, USA). The lengths of the lines indicate the reciprocal of the sequence similarities.

FIG. 17. Structure of lipophilic GABA transport inhibitors and their parent compounds.

FIG. 18. Potency of Tiagabine at cloned GABA transporters. Cell lines expressing hGAT-1 (○), rGAT-2 (□),

hGAT-3 (Δ) and hBGT-1 ($\diamond$) were incubated with [^3H]GABA and the indicated concentrations of Tiagabine. Data show specific uptake, expressed as percent of uptake in the absence of inhibitor. Standard deviations, omitted for clarity, were $\leq 15\%$. This experiment was repeated with similar results.

FIG. 19A. Morphology of neuronal cell cultures. Cultures were prepared as described in Methods. Neuronal cultures were stained with an anti-neurofilament antibody (Boehringer-Mannheim, Indianapolis, Ind.) and visualized with horseradish peroxidase.

FIG. 19B. Morphology of astrocyte cell cultures. Type 1 astrocyte cultures were stained with cresyl violet.

FIG. 19C. Morphology of astrocyte cell cultures. O-2A/Type 2 astrocyte cultures were stained with cresyl violet.

FIG. 20. NNC-711 inhibition of GABA transport in neuronal cultures. Neuronal cultures were incubated with [^3H]GABA and the indicated concentrations of NNC-711, as described in Methods. Data show specific uptake, expressed as percent of uptake in the absence of inhibitor. Data are from a single experiment that was repeated at least three times with similar results.

FIG. 21. NNC-711 inhibition of GABA transport in O-2A/Type 2 astrocytic cultures. O-2A/Type 2 astrocytic cultures were incubated with [^3H]GABA and the indicated concentrations of NNC-711, as described in Methods. Data show specific uptake, expressed as percent of uptake in the absence of inhibitor. Data are the mean of three experiments.

FIG. 22. NNC-711 inhibition of GABA transport in brain aggregates. Rat brain aggregates were incubated with [^3H]GABA and the indicated concentrations of NNC 711, as described in Methods. Data show specific uptake, expressed as percent of uptake in the absence of inhibitor. Data are from a single experiment that was repeated three times with similar results.

FIGS. 23A–23J. Localization of GABA transporter mRNAs. Northern blot analysis of GAT-1 (≈ 4.2 Kb), GAT-2 (≈ 2.4 Kb), GAT-3 (≈ 4.7 Kb), rBGT-1 (≈ 2.4 Kb), and TAUT mRNA (≈ 6.2 Kb). Total RNA from neuronal (lane 1), Type 1 astrocyte (lane 2), and O-2A/Type 2 astrocyte (lane 3) cultures, was separated by formaldehyde/agarose gel electrophoresis, blotted, and hybridized at high stringency with ^{32}P -labeled transporter cDNAs (see Methods). The autoradiograms were developed after 5 days, with the exception of TAUT (overnight). Corresponding autoradiograms representing cyclophilin (1B15) hybridization are shown at right (45 minute exposure). Identical samples of neurons, Type 1 astrocytes, and O-2A/Type 2 astrocytes were used for each of five blots shown. Note that the levels of rBGT-1 mRNA in astrocytes in the experiment shown are lower than average (see Table 7 for quantitation).

FIG. 24A. In situ hybridization localization of GAT-1 mRNA in the periventricular hypothalamus of the rat brain. In sections stained with cresyl violet, silver grains indicative of GAT-1 hybridization signal tend to be clustered over large, pale stained cells (large arrows) which appear to be neurons.

FIG. 24B. In situ hybridization localization of GAT-3 mRNA in the periventricular hypothalamus of the rat brain. Silver grains indicative of GAT-3 hybridization signal are most often observed over small, darkly stained cells (small arrows) The dark staining is typical of glial cells which are GFAP-immunoreactive (small arrows in FIG. 24C), suggesting that the GAT-3 mRNA is localized to glial cells in some areas of the brain.

FIG. 24C. Immunocytochemical localization of glial fibrillary acidic protein (GFAP) in the periventricular hypo-

thalamus of the rat brain. The dark staining is typical of glial cells which are GFAP-immunoreactive (small arrows).

DETAILED DESCRIPTION OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding a mammalian GABA transporter. This invention also provides an isolated nucleic acid molecule encoding a mammalian taurine transporter. This invention further provides an isolated nucleic acid molecule encoding a human GABA transporter. As used herein, the term "isolated nucleic acid molecule" means a non-naturally occurring nucleic acid molecule that is, a molecule in a form which does not occur in nature. Examples of such an isolated nucleic acid molecule are an RNA, cDNA, or isolated genomic DNA molecule encoding a mammalian GABA, or mammalian taurine transporter. As used herein, "GABA transporter" means a molecule which, under physiologic conditions, is substantially specific for the neurotransmitter GABA, is saturable, of high affinity for GABA ($K_m=4 \mu M$), and exhibits pharmacological properties distinct from the neuronal GABA transporter. As used herein, "taurine transporter" means a molecule which, under physiologic conditions, is substantially specific for the neurotransmitter taurine, is saturable, and of high affinity for taurine. One embodiment of this invention is an isolated murine nucleic acid molecule encoding a GABA or taurine transporter. Such a molecule may have coding sequences substantially the same as the coding sequences shown in FIGS. 1A-1D, 2A-2D or 3A-3D. The DNA molecules of FIGS. 1A-1D (Sequence I.D. No. 1) and 2A-2D (Seq I.D. No.3) encode the sequence of the mammalian GABA transporter genes. The DNA molecule of FIGS. 3A-3D (Sequence I.D. No. 5) encodes the sequence of a mammalian taurine transporter gene. Another preferred embodiment of this invention is an isolated human nucleic acid molecule encoding a human GABA transporter. Such a molecule may have coding sequences substantially the same as the coding sequences shown in FIGS. 14A-14B and 15A-15D. The DNA molecules of FIGS. 14A-14B (Sequence I.D. No.7) and 15A-15D (Sequence I.D. No. 9) encode the sequences of human GABA transporter genes. Another preferred embodiment of this invention is an isolated nucleic acid molecule encoding a human taurine transporter. Such a molecule may have coding sequences substantially similar to the sequence shown in FIGS. 3A-3D. One means of isolating a mammalian GABA or a mammalian taurine transporter is to probe a mammalian genomic library with a natural or artificially designed DNA probe, using methods well known in the art. In the preferred embodiment of this invention, the mammalian GABA and mammalian taurine transporter are human proteins and the nucleic acid molecules encoding them are isolated from a human cDNA library or a human genomic DNA library. DNA probes derived from the rat GABA transporter genes rB14b and K438-rB8b, and DNA probes derived from the rat taurine transporter gene rB16a are useful probes for this purpose. DNA and cDNA molecules which encode mammalian GABA or mammalian taurine transporters are used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic clones by the screening of cDNA or genomic DNA libraries, by methods described in more detail below. Transcriptional regulatory elements from the 5' untranslated region of the isolated clone, and other stability, processing, transcription, translation, and tissue specificity determining regions from the 3' and 5' untranslated regions of the isolated gene are thereby obtained.

This invention provides a method for obtaining an isolated nucleic acid molecule encoding a human taurine transporter which comprises using oligonucleotide primers based on the nucleic acid sequence coding for a mammalian taurine receptor and the polymerase chain reaction (PCR) to detect the presence of the nucleic acid molecule coding for the taurine transporter in a human cDNA library. PCR is carried out at reduced annealing temperatures to allow for mismatches between the nucleic acid sequences encoding the rat taurine transporter and nucleic acid sequences encoding the human taurine transporter. Amplified DNA sequences encoding a human taurine transporter are detected by hybridization at reduced hybridization stringency with radiolabelled cDNA encoding the mammalian taurine receptor. A human cDNA library identified by the above method to contain a nucleic acid molecule encoding the human taurine transporter is then screened at low hybridization stringency with the same cDNA probe encoding the mammalian taurine receptor to isolate a cDNA clone encoding a human taurine transporter. A cDNA sequence from the resulting clone can then be used to additionally screen a human cDNA or human genomic DNA library to obtain the entire sequence of the human homologue of the mammalian taurine transporter. Primers used in the polymerase chain reaction to initially screen human cDNA libraries to identify human cDNA libraries containing clones encoding a human taurine receptor may be composed of a plurality of degenerate primers based on the sequence of the mammalian taurine transporter. The methods of synthesizing primers, of screening cDNA libraries by PCR to identify libraries containing a cDNA clone encoding the protein of interest are well known by one of skill in the art and examples of this method for obtaining a cDNA clone encoding the human homologue of mammalian transporter are further given below. These same methods can be used to isolate cDNA and genomic DNAs encoding additional mammalian or human GABA transporter subtypes or taurine transporter subtypes encoded by different genes or encoded by the same gene and generated by alternative splicing of the RNA or rearrangement of the genomic DNA.

This invention provides an isolated nucleic acid molecule which has been so mutated as to be incapable of encoding a molecule having normal transporter activity, and not expressing native transporter. An example of a mutated nucleic acid molecule provided by this invention is an isolated nucleic acid molecule which has an in-frame stop codon inserted into the coding sequence such that the transcribed RNA is not translated into a protein having normal transporter activity.

This invention further provides a cDNA molecule encoding a mammalian GABA transporter, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in FIGS. 1A-1D or 2A-2D. (Sequence I.D. Nos. 1 or 3). This invention also provides a cDNA molecule encoding a mammalian taurine transporter, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in FIGS. 3A-3D. (Sequence I.D. No. 5). This invention also provides a cDNA molecule encoding a human GABA transporter, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in FIGS. 14A-14B (Sequence I.D. No. 7) and 15A-15D (Sequence I.D. No. 9). These molecules and their equivalents were obtained by the means described above.

This invention also provides an isolated protein which is a mammalian GABA transporter. This invention further provides an isolated protein which is a mammalian taurine

transporter. In one embodiment of this invention, the protein is a murine GABA transporter protein having an amino acid sequence substantially similar to the amino acid sequence shown in FIGS. 1A–1D (Seq. I.D. Nos. 1 and 2) or 2A–2D (Seq. I.D. Nos. 3 and 4). In another embodiment of this invention, the protein is a murine taurine transporter protein having an amino acid sequence substantially similar to the amino acid sequence shown in FIGS. 3A–3D (Seq. I.D. Nos. 5 and 6). In a preferred embodiment of this invention, the protein is a human GABA transporter protein having an amino acid sequence substantially the same as the sequence shown in FIGS. 14A–14B (Sequence I.D. Nos. 7 and 8) and FIGS. 15A–15D (Sequence I.D. Nos. 9 and 10). Another preferred embodiment of this invention, the protein is a human taurine transporter protein having an amino acid sequence substantially similar to the amino acid sequence shown in FIGS. 3A–3D (Seq. I.D. Nos. 5 and 6). As used herein, the term “isolated protein” is intended to encompass a protein molecule free of other cellular components. One means for obtaining an isolated GABA or taurine transporter is to express DNA encoding the transporter in a suitable host, such as a bacterial, yeast, or mammalian cell, using methods well known to those skilled in the art, and recovering the transporter protein after it has been expressed in such a host, again using methods well known in the art. The transporter may also be isolated from cells which express it, in particular from cells which have been transfected with the expression vectors described below in more detail.

This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a mammalian GABA transporter. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a mammalian taurine transporter. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human GABA transporter. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human taurine transporter.

Examples of vectors are viruses such as bacteriophages (such as phage lambda), cosmids, plasmids (such as pUC18, available from Pharmacia, Piscataway, N.J.), and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known to those skilled in the art. Examples of such plasmids are plasmids comprising cDNA having a coding sequence substantially the same as: the coding sequence shown in FIGS. 1A–1D (Seq. I.D. No. 1) and designated clone pEVJB-rB14b deposited Feb. 7, 1992 under ATCC Accession No. 75203, the coding sequence shown in FIGS. 2A–2D (Seq. I.D. No. 3) and a portion of which is designated clone pEVJB-rB8b deposited Feb. 7, 1992 under ATCC Accession No. 75201, the coding sequence shown in FIGS. 3A–3D (Seq. I.D. No. 5) and designated pEVJB-rB16a deposited Feb. 7, 1992 under ATCC Accession No. 75202, the coding sequence shown in FIGS. 14A–14B (Sequence I.D. No. 7) designated PBluescript-hHE7a and pBluescript-hS3a and deposited Oct. 8, 1992 under ATCC Accession Nos. 75322 and 75323, respectively, or the coding sequence shown in FIGS. 15A–15D (SEQ. I.D. No. 9) and designated pcEXV-hGAT-3 and deposited Oct. 8, 1992 under ATCC Accession No. 75324. Alternatively, to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with a ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA,

which is then digested with the restriction enzyme which cuts at that site. Other means are also available.

This invention also provides vectors comprising a DNA molecule encoding a mammalian GABA transporter and vectors comprising a DNA molecule encoding a mammalian taurine transporter, adapted for expression in a bacterial cell, a yeast cell, insect, or a mammalian cell which additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, insect, or mammalian cells so located relative to the DNA encoding a mammalian GABA transporter or to the DNA encoding a mammalian taurine transporter as to permit expression thereof. DNA having coding sequences substantially the same as the coding sequence shown in FIGS. 1A–1D or FIGS. 2A–2D may usefully be inserted into the vectors to express mammalian GABA transporters. DNA having coding sequences substantially the same as the coding sequence shown in FIGS. 3A–3C may usefully be inserted into the vectors to express mammalian taurine transporters. This invention also provides vectors comprising a DNA molecule encoding a human GABA transporter adapted for expression in a bacterial cell, a yeast cell, insect, or a mammalian cell which additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, insect, or mammalian cells so located relative to the DNA encoding a human GABA transporter as to permit expression thereof. DNA having coding sequences substantially the same as the coding sequence shown in FIGS. 14A–14B and 15A–15D may usefully be inserted into the vectors to express human GABA transporters. This invention also provides vectors comprising a DNA molecule encoding a human taurine transporter adapted for expression in a bacterial cell, a yeast cell, insect, or a mammalian cell which additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, insect, or mammalian cells so located relative to the DNA encoding a human taurine transporter as to permit expression thereof. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Maniatis, et al., *Molecular Cloning*, Cold Spring Harbor Laboratory, 1982). Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the transporter. Certain uses for such cells are described in more detail below.

In one embodiment of this invention a plasmid is adapted for expression in a bacterial, yeast, or, in particular, a mammalian cell wherein the plasmid comprises a DNA molecule encoding a mammalian GABA transporter or a DNA molecule encoding a mammalian taurine transporter and the regulatory elements necessary for expression of the DNA in the bacterial, yeast, or mammalian cell so located relative to the DNA encoding a mammalian GABA transporter or to the DNA encoding a mammalian taurine transporter as to permit expression thereof. In another embodiment of this invention a plasmid is adapted for expression in a bacterial, yeast, or, in particular, a mammalian cell wherein the plasmid comprises a DNA molecule encoding a human

GABA transporter or human taurine transporter and the regulatory elements necessary for expression of the DNA in the bacterial, yeast, or mammalian cell so located relative to the DNA encoding a human GABA transporter or human taurine transporter as to permit expression thereof. Suitable plasmids may include, but are not limited to plasmids adapted for expression in a mammalian cell, e.g., EVJB or EXV. Examples of such plasmids adapted for expression in a mammalian cell are plasmids comprising cDNA having coding sequences substantially the same as the coding sequence shown in FIGS. 1A–1D, a portion of 2A–2D, 3A–3D, 14A–14B and 15A–15D and the regulatory elements necessary for expression of the DNA in the mammalian cell. These plasmids have been designated pEVJB-rB14b deposited under ATCC Accession No.75203, pEVJB-rB8b deposited under ATCC Accession No.75201, pEVJB-rB16a deposited under ATCC Accession No.75202, pBluescript-hHE7a and pBluescript-hS3a deposited under ATCC Accession Nos. 75322 and 75323, and pcEXV-hGAT-3 deposited under ATCC accession No. 75324, respectively. Those skilled in the art will readily appreciate that numerous plasmids adapted for expression in a mammalian cell which comprise DNA encoding a mammalian GABA transporter, a mammalian taurine transporter, a human GABA transporter or human taurine transporter and the regulatory elements necessary to express such DNA in the mammalian cell may be constructed utilizing existing plasmids and adapted as appropriate to contain the regulatory elements necessary to express the DNA in the mammalian cell. The plasmids may be constructed by the methods described above for expression vectors and vectors in general, and by other methods well known in the art. The deposits discussed supra were made pursuant to, and in satisfaction of, the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852.

This invention provides a mammalian cell comprising a DNA molecule encoding a mammalian GABA transporter or a DNA molecule encoding a mammalian taurine transporter, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a mammalian GABA transporter or a DNA encoding a mammalian taurine transporter and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a mammalian transporter as to permit expression thereof. This invention also provides a mammalian cell comprising a DNA molecule encoding a human GABA transporter or a human taurine transporter, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a human GABA transporter or DNA encoding a human taurine transporter and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a human transporter as to permit expression thereof. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk⁻ cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, or DNA encoding these transporters may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a mammalian GABA transporter, encod-

ing a mammalian taurine transporter or encoding a human GABA transporter.

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian GABA transporter, for example with a coding sequence included within the sequences shown in FIGS. 1A–1D and 2A–2D. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a taurine transporter, for example with a coding sequence included within the sequence shown in FIGS. 3A–3D. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human GABA transporter, for example with a coding sequence included within the sequence shown in FIGS. 14A–14B and 15A–15D. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human taurine transporter, for example with a coding sequence substantially similar to the coding sequence included within the sequence shown in FIGS. 3A–3D. As used herein, the phrase “specifically hybridizing” means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. As used herein, a “unique sequence” is a sequence specific to only the nucleic acid molecules encoding the subject transporter. An unique sequence of a nucleic acid molecule encoding the GAT-2 human transporter would be a sequence of nucleotides that can only be found in the GAT-2 human transporter. A probe which specifically hybridizes with a unique sequence within a subject transporter is capable of specifically hybridizing with that unique sequence and no other sequence. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. Detection of nucleic acid encoding a mammalian GABA transporter, mammalian taurine transporter, human GABA transporter or human taurine transporter is useful as a diagnostic test for any disease process in which levels of expression of the corresponding GABA or taurine transporter are altered. DNA probe molecules are produced by insertion of a DNA molecule which encodes the mammalian GABA transporter, the mammalian taurine transporter, the human GABA transporter or the human taurine transporter or fragments thereof into suitable vectors, such as plasmids or bacteriophages, followed by insertion into suitable bacterial host cells and replication and harvesting of the DNA probes, all using methods well known in the art. For example, the DNA may be extracted from a cell lysate using phenol and ethanol, digested with restriction enzymes corresponding to the insertion sites of the DNA into the vector (discussed above), electrophoresed, and cut out of the resulting gel. Examples of such DNA molecules are shown in FIGS. 1A–1D, 2A–2D, 3A–3D, 14A–14B and 15A–15D. The probes are useful for ‘in situ’ hybridization or in order to locate tissues which express this gene family, or for other hybridization assays for the presence of these genes or their

mRNA in various biological tissues. In addition, synthesized oligonucleotides (produced by a DNA synthesizer) complementary to the sequence of a DNA molecule which encodes a mammalian GABA transporter or a mammalian taurine transporter or complementary to the sequence of a DNA molecule which encodes a human GABA transporter or human taurine transporter, are useful as probes for these genes, for their associated mRNA, or for the isolation of related genes by homology screening of genomic or cDNA libraries, or by the use of amplification techniques such as the Polymerase Chain Reaction.

This invention also provides a method of detecting expression of a GABA transporter on the surface of a cell by detecting the presence of mRNA coding for a GABA transporter. This invention also provides a method of detecting expression of a taurine transporter on the surface of the cell by detecting the presence of mRNA coding for a taurine transporter. This invention further provides a method of detecting the expression of a human GABA or human taurine transporter on the surface of the cell by detecting the presence of mRNA coding for the corresponding GABA or taurine transporter. These methods comprise obtaining total mRNA from the cell using methods well known in the art and contacting the mRNA so obtained with a nucleic acid probe as described hereinabove, under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the transporter by the cell. Hybridization of probes to target nucleic acid molecules such as mRNA molecules employs techniques well known in the art. However, in one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the mRNA is isolated from the extract using a column which binds the poly-A tails of the mRNA molecules (48). The mRNA is then exposed to radioactively labeled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

This invention provides an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes a mammalian GABA transporter so as to prevent translation of the mammalian GABA transporter. This invention also provides an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes a mammalian taurine transporter so as to prevent translation of the mammalian taurine transporter. This invention provides an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes a human GABA transporter so as to prevent translation of the human GABA transporter. This invention also provides an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes a human taurine transporter so as to prevent translation of the human taurine transporter. As used herein, the phrase "binding specifically" means the ability of an antisense oligonucleotide to (a) bind a nucleic acid sequence complementary or substantially complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs, and (b) not bind nucleic acid sequences other than the nucleic acid sequence encoding the subject transporter. The antisense oligonucleotide may have a sequence capable of binding

specifically with any sequences of the cDNA molecules whose sequences are shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B and 15A-15D. A particular example of an antisense oligonucleotide is an antisense oligonucleotide comprising chemical analogues of nucleotides.

This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a mammalian GABA transporter by passing through a cell membrane and binding specifically with mRNA encoding a mammalian GABA transporter in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a mammalian taurine transporter by passing through a cell membrane and binding specifically with mRNA encoding a mammalian taurine transporter in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a human GABA transporter by passing through a cell membrane and binding specifically with mRNA encoding a human GABA transporter in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a human taurine transporter by passing through a cell membrane and binding specifically with mRNA encoding a human taurine transporter in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The oligonucleotide may be coupled to a substance which inactivates mRNA, such as a ribozyme. The pharmaceutically acceptable hydrophobic carrier capable of passing through cell membranes may also comprise a structure which binds to a transporter specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind a cell-type specific transporter, for example an insulin molecule, which would target pancreatic cells. DNA molecules having coding sequences substantially the same as the coding sequence shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B or 15A-15D may be used as the oligonucleotides of the pharmaceutical composition.

This invention also provides a method of treating abnormalities which are alleviated by reduction of expression of a GABA transporter. This method comprises administering to a subject an effective amount of the pharmaceutical composition described above effective to reduce expression of the GABA transporter by the subject. This invention further provides a method of treating an abnormal condition related to GABA transporter activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to reduce expression of the GABA transporter by the subject. Examples of such abnormal conditions are epilepsy and generalized anxiety. This invention also provides a method of treating abnormalities which are alleviated by reduction of expression of

a taurine transporter. This method comprises administering to a subject an effective amount of the pharmaceutical composition described above effective to reduce expression of the taurine transporter by the subject.

This invention further provides a method of treating an abnormal condition related to taurine transporter activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to reduce expression of the taurine transporter by the subject. Examples of such abnormal conditions are epilepsy, migraine, and ischemia.

Antisense oligonucleotide drugs inhibit translation of mRNA encoding these transporters. Synthetic antisense oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding a GABA transporter or to mRNA encoding a taurine transporter and inhibit translation of mRNA and are useful as drugs to inhibit expression of GABA transporter genes or taurine transporter genes in patients. This invention provides a means to therapeutically alter levels of expression of mammalian GABA or taurine transporters by the use of a synthetic antisense oligonucleotide drug (SAOD) which inhibits translation of mRNA encoding these transporters. Synthetic antisense oligonucleotides, or other antisense chemical structures designed to recognize and selectively bind to mRNA, are constructed to be complementary to portions of the nucleotide sequences shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B or 15A-15D of DNA, RNA or of chemically modified, artificial nucleic acids. The SAOD is designed to be stable in the blood stream for administration to patients by injection, or in laboratory cell culture conditions, for administration to cells removed from the patient. The SAOD is designed to be capable of passing through cell membranes in order to enter the cytoplasm of the cell by virtue of physical and chemical properties of the SAOD which render it capable of passing through cell membranes (e.g., by designing small, hydrophobic SAOD chemical structures) or by virtue of specific transport systems in the cell which recognize and transport the SAOD into the cell. In addition, the SAOD can be designed for administration only to certain selected cell populations by targeting the SAOD to be recognized by specific cellular uptake mechanisms which bind and take up the SAOD only within certain selected cell populations. For example, the SAOD may be designed to bind to a transporter found only in a certain cell type, as discussed above. The SAOD is also designed to recognize and selectively bind to the target mRNA sequence, which may correspond to a sequence contained within the sequences shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B or 15A-15D by virtue of complementary base pairing to the mRNA. Finally, the SAOD is designed to inactivate the target mRNA sequence by any of three mechanisms: 1) by binding to the target mRNA and thus inducing degradation of the mRNA by intrinsic cellular mechanisms such as RNase I digestion, 2) by inhibiting translation of the mRNA target by interfering with the binding of translation-regulating factors or of ribosomes, or 3) by inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups, which either degrade or chemically modify the target mRNA. Synthetic antisense oligonucleotide drugs have been shown to be capable of the properties described above when directed against mRNA targets (11,76). In addition, coupling of ribozymes to antisense oligonucleotides is a promising strategy for inactivating target mRNA (60). An SAOD serves as an effective therapeutic agent if it is designed to be administered to a patient by injection, or if the patient's target cells are

removed, treated with the SAOD in the laboratory, and replaced in the patient. In this manner, an SAOD serves as a therapy to reduce transporter expression in particular target cells of a patient, in any clinical condition which may benefit from reduced expression of GABA or taurine transporters.

This invention provides an antibody directed to the mammalian GABA transporter. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a mammalian GABA transporter present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the mammalian GABA transporter included in the amino acid sequence shown in FIGS. 1A-1D or 2A-2D. This invention provides an antibody directed to the mammalian taurine transporter. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a mammalian taurine transporter present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the mammalian taurine transporter included in the amino acid sequence shown in FIGS. 3A-3D. This invention provides an antibody directed to a human GABA transporter. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human GABA transporter present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human GABA transporter included in the amino acid sequence shown in FIGS. 14A-14B and 15A-15D. This invention provides an antibody directed to a human taurine transporter. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human taurine transporter present on the surface of a cell, the epitope having an amino acid sequence substantially similar to the amino acid sequence for a cell surface epitope of the mammalian taurine transporter shown in FIGS. 3A-3D. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer which forms the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Therefore antibodies to the hydrophilic amino acid sequences shown in FIGS. 1A-1D or 2A-2D will bind to a surface epitope of a mammalian GABA transporter, and antibodies to the hydrophilic amino acid sequences shown in FIGS. 3A-3D will bind to a surface epitope of a mammalian taurine transporter, as described. Antibodies to the hydrophilic amino acid sequences shown in FIGS. 14A-14B or 15A-15D will bind to a surface epitope of a human GABA transporter. Antibodies directed to conserved hydrophilic amino acid sequences specific to a mammalian taurine transporter will bind to a surface epitope of a human taurine transporter. Antibodies directed to mammalian or human transporters may be serum-derived or monoclonal and are prepared using methods well known in the art. For example, monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Cells such as NIH3T3 cells or Ltk⁻ cells may be used as immunogens to raise such an antibody. Alternatively, synthetic peptides may be prepared using commercially available machines and the amino acid sequences shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B and 15A-15D. As a still further alternative, DNA,

such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen. These antibodies are useful to detect the presence of mammalian transporters encoded by the isolated DNA, or to inhibit the function of the transporters in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

This invention also provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of the mammalian transporter, effective to block binding of naturally occurring substrates to the transporter, and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a mammalian GABA transporter present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the mammalian GABA transporter included in the amino acid sequences shown in FIGS. 1A–1D and 2A–2D is useful for this purpose. A monoclonal antibody directed to an epitope of a mammalian taurine transporter present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the mammalian taurine transporter included in the amino acid sequence shown in FIGS. 3A–3D is also useful for this purpose.

This invention also provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of the human transporter, effective to block binding of naturally occurring substrates to the transporter, and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human GABA transporter present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human GABA transporter included in the amino acid sequences shown in FIGS. 14A–14B or 15A–15D is useful for this purpose.

This invention also provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of a human taurine transporter, effective to block binding of naturally occurring substrates to the human taurine transporter, and a pharmaceutically acceptable carrier. A monoclonal antibody directed to a conserved epitope specific to a mammalian taurine transporter present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the mammalian taurine transporter included in the amino acid sequence shown in FIGS. 3A–3D is useful for this purpose.

This invention also provides a method of treating abnormalities in a subject which are alleviated by reduction of expression of a mammalian transporter which comprises administering to the subject an effective amount of the pharmaceutical composition described above effective to block binding of naturally occurring substrates to the transporter and thereby alleviate abnormalities resulting from overexpression of a mammalian transporter. Binding of the antibody to the transporter prevents the transporter from functioning, thereby neutralizing the effects of overexpression. The monoclonal antibodies described above are both useful for this purpose. This invention additionally provides a method of treating an abnormal condition related to an excess of transporter activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to block binding of naturally occurring substrates to the transporter and thereby alleviate the abnormal condition. Some examples of abnormal conditions associated with excess GABA transporter activity are

epilepsy and generalized anxiety. Excess taurine transporter activity associated disorders are epilepsy, migraine, and ischemia.

This invention provides methods of detecting the presence of a GABA or a taurine transporter on the surface of a cell which comprises contacting the cell with an antibody directed to the mammalian GABA transporter or an antibody directed to the mammalian taurine transporter, under conditions permitting binding of the antibody to the transporter, detecting the presence of the antibody bound to the cell, and thereby the presence of the mammalian GABA transporter or the presence of the taurine transporter on the surface of the cell. Such methods are useful for determining whether a given cell is defective in expression of GABA transporters or is defective in expression of taurine transporters on the surface of the cell. Bound antibodies are detected by methods well known in the art, for example by binding fluorescent markers to the antibodies and examining the cell sample under a fluorescence microscope to detect fluorescence on a cell indicative of antibody binding. The monoclonal antibodies described above are useful for this purpose.

This invention provides a transgenic nonhuman mammal expressing DNA encoding a mammalian GABA transporter and a transgenic nonhuman mammal expressing DNA encoding a mammalian taurine transporter. This invention further provides a transgenic nonhuman mammal expressing DNA encoding a human GABA transporter and a transgenic nonhuman mammal expressing DNA encoding a human taurine transporter. This invention also provides a transgenic nonhuman mammal expressing DNA encoding a mammalian GABA transporter so mutated as to be incapable of normal transporter activity, and not expressing native GABA transporter and a transgenic nonhuman mammal expressing DNA encoding a mammalian taurine transporter so mutated as to be incapable of normal transporter activity, and not expressing native taurine transporter. This invention further provides a transgenic nonhuman mammal expressing DNA encoding a human GABA transporter so mutated as to be incapable of normal transporter activity, and not expressing native GABA transporter and a transgenic nonhuman mammal expressing DNA encoding a human taurine transporter so mutated as to be incapable of normal transporter activity, and not expressing native taurine transporter.

This invention provides a transgenic nonhuman mammal whose genome comprises DNA encoding a mammalian GABA transporter so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a GABA transporter and which hybridizes to mRNA encoding a GABA transporter thereby reducing its translation and a transgenic nonhuman mammal whose genome comprises DNA encoding a mammalian taurine transporter so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a taurine transporter and which hybridizes to mRNA encoding a taurine transporter thereby reducing its translation. This invention further provides a transgenic nonhuman mammal whose genome comprises DNA encoding a human GABA transporter so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a GABA transporter and which hybridizes to mRNA encoding a GABA transporter thereby reducing its translation and a transgenic nonhuman mammal whose genome comprises DNA encoding a human taurine transporter so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a taurine transporter and which hybridizes to mRNA encoding a taurine transporter thereby reducing its translation. The DNA may additionally comprise an inducible promoter or

additionally comprise tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of DNA are DNA or cDNA molecules having a coding sequence substantially the same as the coding sequences shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B and 15A-15D. An example of a transgenic animal is a transgenic mouse. Examples of tissue specificity-determining regions are the metallothionein promoter (46, 83) and the L7 promoter (84).

Animal model systems which elucidate the physiological and behavioral roles of mammalian transporters are produced by creating transgenic animals in which the expression of a transporter is either increased or decreased, or the amino acid sequence of the expressed transporter protein is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a mammalian transporter or homologous animal versions of these genes, by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal (24) or 2) Homologous recombination (7,82) of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these transporters. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native transporter but does express, for example, an inserted mutant transporter, which has replaced the native transporter in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added transporters, resulting in overexpression of the transporter.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (24). DNA or cDNA encoding a mammalian transporter is purified from a vector (such as plasmids EVJB-rB14b, EVJB-rB8b, or EVJB-rB16a described above) by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipette puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Since the normal action of transporter-specific drugs is to activate or to inhibit the transporter, the transgenic animal model systems described above are useful for testing the biological activity of drugs directed against these transporters even before such drugs become available. These animal model systems are useful for predicting or evaluating possible therapeutic applications of drugs which activate or

inhibit these transporters by inducing or inhibiting expression of the native or trans-gene and thus increasing or decreasing expression of normal or mutant transporters in the living animal. Thus, a model system is produced in which the biological activity of drugs directed against these transporters are evaluated before such drugs become available. The transgenic animals which over or under produce the transporter indicate by their physiological state whether over or under production of the transporter is therapeutically useful. It is therefore useful to evaluate drug action based on the transgenic model system. One use is based on the fact that it is well known in the art that a drug such as an antidepressant acts by blocking neurotransmitter uptake, and thereby increases the amount of neurotransmitter in the synaptic cleft. The physiological result of this action is to stimulate the production of less transporter by the affected cells, leading eventually to underexpression. Therefore, an animal which underexpresses transporter is useful as a test system to investigate whether the actions of such drugs which result in under expression are in therapeutic. Another use is that if overexpression is found to lead to abnormalities, then a drug which down-regulates or acts as an antagonist to the transporter is indicated as worth developing, and if a promising therapeutic application is uncovered by these animal model systems, activation or inhibition of the GABA transporter is achieved therapeutically either by producing agonist or antagonist drugs directed against these GABA transporters or by any method which increases or decreases the expression of these transporters in man.

Further provided by this invention is a method of determining the physiological effects of expressing varying levels of mammalian transporters which comprises producing a transgenic nonhuman animal whose levels of mammalian transporter expression are varied by use of an inducible promoter which regulates mammalian transporter expression. This invention also provides a method of determining the physiological effects of expressing varying levels of mammalian transporters which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of mammalian transporter. Such animals may be produced by introducing different amounts of DNA encoding a mammalian transporter into the oocytes from which the transgenic animals are developed.

This invention provides a method of determining the physiological effects of expressing varying levels of human transporters which comprises producing a transgenic nonhuman animal whose levels of human transporter expression are varied by use of an inducible promoter which regulates transporter expression. This invention also provides a method of determining the physiological effects of expressing varying levels of human transporters which comprises producing a panel of transgenic nonhuman animals each expressing a different amount of the human transporter. Such animals may be produced by introducing different amounts of DNA encoding a human transporter into the oocytes from which the transgenic animals are developed.

This invention also provides a method for identifying a substance capable of alleviating abnormalities resulting from overexpression of a mammalian transporter comprising administering the substance to a transgenic nonhuman mammal expressing at least one artificially introduced DNA molecule encoding a mammalian transporter and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of a mammalian transporter. This invention also provides a method for iden-

tifying a substance capable of alleviating abnormalities resulting from overexpression of a human transporter comprising administering the substance to a transgenic nonhuman mammal expressing at least one artificially introduced DNA molecule encoding a human transporter and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of a human transporter. As used herein, the term "substance" means a compound or composition which may be natural, synthetic, or a product derived from screening. Examples of DNA molecules are DNA or cDNA molecules having a coding sequence substantially the same as the coding sequences shown in FIGS. 1A-1D, 2A-2D, 3A-3D, 14A-14B and 15A-15D.

This invention provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of GABA transporter and a pharmaceutically acceptable carrier. This invention also provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of taurine transporter and a pharmaceutically acceptable carrier. This invention further provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human GABA or human taurine transporter and a pharmaceutically acceptable carrier.

This invention also provides a method for treating the abnormalities resulting from overexpression of a mammalian transporter which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from overexpression of a mammalian transporter. This invention further provides a method for treating the abnormalities resulting from overexpression of a human GABA or human taurine transporter which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from overexpression of a human GABA or taurine transporter.

This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a mammalian transporter comprising administering the substance to the transgenic nonhuman mammal described above which expresses only nonfunctional mammalian transporter and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of a mammalian transporter. This invention further provides a method for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human GABA or human taurine transporter comprising administering the substance to the transgenic nonhuman mammal described above which expresses only nonfunctional human GABA or human taurine transporter and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of a human GABA or human taurine transporter.

This invention also provides a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of transporter and a pharmaceutically acceptable carrier. This invention also provides a pharmaceutical composition comprising an amount of a substance effective to alleviate

abnormalities resulting from underexpression of a human GABA or human taurine transporter and a pharmaceutically acceptable carrier.

This invention provides a method for treating the abnormalities resulting from underexpression of a mammalian transporter which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from underexpression of a mammalian transporter. This invention further provides a method for treating the abnormalities resulting from underexpression of a human GABA or human taurine transporter which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from underexpression of a human GABA or human taurine transporter.

This invention provides a method for diagnosing a predisposition to a disorder associated with the expression of a specific mammalian transporter allele which comprises: a) obtaining DNA of subjects suffering from the disorder; b) performing a restriction digest of the DNA with a panel of restriction enzymes; c) electrophoretically separating the resulting DNA fragments on a sizing gel; d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to DNA encoding a mammalian transporter and labeled with a detectable marker; e) detecting labeled bands which have hybridized to the DNA encoding a mammalian transporter labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; f) preparing DNA obtained for diagnosis by steps a-e; and g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step e and the DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and thereby to diagnose predisposition to the disorder if the patterns are the same. This method may also be used to diagnose a disorder associated with the expression of a specific mammalian transporter allele.

This invention provides a method for diagnosing a predisposition to a disorder associated with the expression of a specific human GABA or human taurine transporter allele which comprises: a) obtaining DNA of subjects suffering from the disorder; b) performing a restriction digest of the DNA with a panel of restriction enzymes; c) electrophoretically separating the resulting DNA fragments on a sizing gel; d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to DNA encoding a human GABA or human taurine transporter and labeled with a detectable marker; e) detecting labeled bands which have hybridized to the DNA encoding a human GABA or human taurine transporter labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; f) preparing DNA obtained for diagnosis by steps a-e; and g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step e and the DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and thereby to diagnose predisposition to the disorder if the patterns are the same. This method may also be used to diagnose a disorder associated with the expression of a specific human GABA or human taurine transporter allele.

This invention provides a method of preparing the isolated transporter which comprises inducing cells to express transporter, recovering the transporter from the resulting cells, and purifying the transporter so recovered. An example of an isolated GABA transporter is an isolated

protein having substantially the same amino acid sequence as the amino acid sequence shown in FIGS. 1A–1D or 2A–2D. An example of an isolated taurine transporter is an isolated protein having substantially the same amino acid sequence shown in FIGS. 3A–3D. This invention further provides a method for preparing an isolated human GABA transporter which comprises inducing cells to express the human GABA transporter, recovering the human GABA transporter from the resulting cells, and purifying the human GABA transporter so recovered. An example of an isolated human GABA transporter is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in FIGS. 14A–14B or 15A–15D. This invention further provides a method for preparing an isolated human taurine transporter which comprises inducing cells to express the human taurine transporter, recovering the human taurine transporter from the resulting cells, and purifying the human taurine transporter so recovered. An example of an isolated human taurine transporter is an isolated protein having an amino acid sequence substantially similar to the amino acid sequence of a mammalian taurine transporter shown in FIGS. 3A–3D. For example, cells can be induced to express transporters by exposure to substances such as hormones. The cells can then be homogenized and the transporter isolated from the homogenate using an affinity column comprising, for example, GABA, taurine, or another substance which is known to bind to the transporter. The resulting fractions can then be purified by contacting them with an ion exchange column, and determining which fraction contains transporter activity or binds anti-transporter antibodies.

This invention provides a method of preparing the isolated mammalian GABA transporter which comprises inserting nucleic acid encoding the mammalian GABA transporter in a suitable vector, inserting the resulting vector in a suitable host cell, recovering the transporter produced by the resulting cell, and purifying the transporter so recovered. An example of an isolated GABA transporter is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in FIGS. 1A–1D or 2A–2D. This invention also provides a method of preparing the isolated mammalian taurine transporter which comprises inserting nucleic acid encoding a mammalian taurine transporter in a suitable vector, inserting the resulting vector in a suitable host cell, recovering the transporter produced by the resulting cell, and purifying the transporter so recovered. This invention also provides a method of preparing the isolated human GABA transporter which comprises inserting nucleic acid encoding the human GABA transporter in a suitable vector, inserting the resulting vector in a suitable host cell, recovering the human GABA transporter produced by the resulting cell, and purifying the human GABA transporter so recovered. These methods for preparing GABA or taurine transporters uses recombinant DNA technology methods well known in the art. For example, isolated nucleic acid encoding GABA or taurine transporter is inserted in a suitable vector, such as an expression vector. A suitable host cell, such as a bacterial cell, or a eukaryotic cell such as a yeast cell, is transfected with the vector. GABA or taurine transporter is isolated from the culture medium by affinity purification or by chromatography or by other methods well known in the art.

This invention provides a method for determining whether a substrate not known to be capable of binding to a mammalian GABA transporter can bind to the mammalian GABA transporter which comprises contacting a mammalian cell comprising a DNA molecule encoding a mamma-

lian GABA transporter with the substrate under conditions permitting binding of substrates known to bind to the transporter, detecting the presence of any of the substrate bound to the transporter, and thereby determining whether the substrate binds to the transporter. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in FIGS. 1A–1D or 2A–2D. This invention provides a method for determining whether a substrate not known to be capable of binding to a mammalian taurine transporter can bind to the mammalian GABA transporter which comprises contacting a mammalian cell comprising a DNA molecule encoding a mammalian taurine transporter with the substrate under conditions permitting binding of substrates known to bind to the transporter, detecting the presence of any of the substrate bound to the transporter, and thereby determining whether the substrate binds to the transporter. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in FIGS. 3A–3D.

This invention also provides a method for determining whether a substrate not known to be capable of binding to a human GABA transporter can bind to a human GABA transporter which comprises contacting a mammalian cell comprising a DNA molecule encoding a human GABA transporter with the substrate under conditions permitting binding of substrates known to bind to the transporter, detecting the presence of any of the substrate bound to the transporter, and thereby determining whether the substrate binds to the transporter. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in FIGS. 14A–14B or 15A–15D. This invention also provides a method for determining whether a substrate not known to be capable of binding to a human taurine transporter can bind to a human taurine transporter which comprises contacting a mammalian cell comprising a DNA molecule encoding a human taurine transporter with the substrate under conditions permitting binding of substrates known to bind to the transporter, detecting the presence of any of the substrate bound to the transporter, and thereby determining whether the substrate binds to the transporter. Preferably, the mammalian cell is nonneuronal in origin. An example of a nonneuronal mammalian cell is a Cos7 cell. The preferred method for determining whether a substrate is capable of binding to the mammalian transporter comprises contacting a transfected nonneuronal mammalian cell (i.e. a cell that does not naturally express any type of transporter, thus will only express such a transporter if it is transfected into the cell) expressing a transporter on its surface, or contacting a membrane preparation derived from such a transfected cell, with the substrate under conditions which are known to prevail, and thus to be associated with, in vivo binding of the substrates to a transporter, detecting the presence of any of the substrate being tested bound to the transporter on the surface of the cell, and thereby determining whether the substrate binds to the transporter. This response system is obtained by transfection of isolated DNA into a suitable host cell. Such a host system might be isolated from pre-existing cell lines, or can be generated by inserting appropriate components into existing cell lines. Such a transfection system provides a complete response system for investigation or assay of the functional activity of mammalian transporters with substrates as described above. Transfection systems are useful as living cell cultures for competitive binding assays between known or candidate drugs and substrates which bind to the transporter and which are labeled by radioactive, spectroscopic or other reagents. Membrane preparations containing the trans-

porter isolated from transfected cells are also useful for these competitive binding assays. A transfection system constitutes a “drug discovery system” useful for the identification of natural or synthetic compounds with potential for drug development that can be further modified or used directly as therapeutic compounds to activate or inhibit the natural functions of the mammalian transporter and/or the human transporter. The transfection system is also useful for determining the affinity and efficacy of known drugs at the mammalian transporter sites and human transporter sites.

This invention provides a method for isolating membranes which comprise GABA or taurine transporters. In a preferred embodiment of the invention, membranes comprising a GABA or taurine transporter are isolated from transfected cells comprising a plasmid vector which further comprises the regulatory elements necessary for the expression of the DNA encoding a GABA or taurine transporter so located relative to the DNA encoding the GABA or taurine transporter as to permit expression thereof. The DNA may have the coding sequence substantially the same as the sequence shown in FIGS. 1A–1D, 2A–2D, 3A–3D, 14A–14B and 15A–15D. The host cell may be a bacterial, yeast, or a mammalian cell. Examples of such cells include the mouse fibroblast cell line NIH3T3, CHO cells, HELA cells, Ltk⁻ cells and Y1 cells. A method for isolating membranes which contain a GABA or taurine transporter comprises preparing a cell lysate from cells expressing the GABA or taurine transporter and isolating membranes from the cell lysate. Methods for the isolation of membranes are well known by one of skill in the art. A method for the isolation of membranes from transfected cells is further described by Branchek et al. (1990). Membranes isolated from transfected cells expressing a GABA or taurine transporter are useful for identifying compounds which may include substrates, drugs or other molecules that specifically bind to a GABA or taurine transporter using radioligand binding methods (Branchek et al. 1990) or other methods described herein. The specificity of the binding of the compound to the transporter may be identified by its high affinity for a particular transporter.

This invention further provides a method for the isolation of vesicles from cells expressing a GABA or taurine transporter. In a preferred embodiment of the invention, vesicles comprising a GABA or taurine transporter are isolated from transfected cells comprising a plasmid vector which further comprises the regulatory elements necessary for the expression of the DNA encoding a GABA or taurine transporter so located relative to the DNA encoding the GABA or taurine transporter as to permit expression thereof. The DNA may have the coding sequence substantially the same as the sequence shown in FIGS. 1A–1D, 2A–2D, 3A–3D, 14A–14B and 15A–15D. A method for the isolation of vesicles is described by Barber and Jamieson (1970) and by Mabeesh et al. (1992). Vesicles comprising a GABA or taurine transporter are useful for assaying and identifying compounds, which may include substrates, drugs or other molecules that enhance or decrease GABA or taurine transporter activity. The compounds may modulate transporter activity by interacting directly with the transporter or by interacting with other cellular components that modulate transporter activity. Vesicles provide an advantage over whole cells in that the vesicles permit one to choose the ionic compositions on both sides of the membrane such that transporter activity and its modulation by can be studied under a variety of controlled physiological or non-physiological conditions. Methods for the assay of transporter activity are well known by one of skill in the art and

are described herein below and by Kannner (1978) and Rudnick (1977).

This invention also provides a method of screening drugs to identify drugs which specifically interact with, and bind to, the mammalian GABA transporter on the surface of a cell which comprises contacting a mammalian cell comprising a DNA molecule encoding a mammalian GABA transporter on the surface of a cell with a plurality of drugs, detecting those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, the mammalian GABA transporter. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in FIGS. 1A–1D or 2A–2D. This invention also provides a method of screening drugs to identify drugs which specifically interact with, and bind to, the mammalian taurine transporter on the surface of a cell which comprises contacting a mammalian cell comprising a DNA molecule encoding a mammalian taurine transporter on the surface of a cell with a plurality of drugs, detecting those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, the mammalian taurine transporter. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in FIGS. 3A–3D. This invention also provides a method of screening drugs to identify drugs which specifically interact with, and bind to, a human GABA transporter on the surface of a cell which comprises contacting a mammalian cell comprising a DNA molecule encoding a human GABA transporter on the surface of a cell with a plurality of drugs, detecting those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, the human GABA transporter. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in FIGS. 14A–14B or 15A–15D. This invention also provides a method of screening drugs to identify drugs which specifically interact with, and bind to, a human taurine transporter on the surface of a cell which comprises contacting a mammalian cell comprising a DNA molecule encoding a human taurine transporter on the surface of a cell with a plurality of drugs, detecting those drugs which bind to the mammalian cell, and thereby identifying drugs which specifically interact with, and bind to, the human taurine transporter. Various methods of detection may be employed. The drugs may be “labeled” by association with a detectable marker substance (e.g., radiolabel or a non-isotopic label such as biotin). Preferably, the mammalian cell is nonneuronal in origin. An example of a nonneuronal mammalian cell is a Cos7 cell. Drug candidates are identified by choosing chemical compounds which bind with high affinity to the expressed transporter protein in transfected cells, using radioligand binding methods well known in the art, examples of which are shown in the binding assays described herein. Drug candidates are also screened for selectivity by identifying compounds which bind with high affinity to one particular transporter subtype but do not bind with high affinity to any other transporter subtype or to any other known transporter site. Because selective, high affinity compounds interact primarily with the target transporter site after administration to the patient, the chances of producing a drug with unwanted side effects are minimized by this approach. This invention provides a pharmaceutical composition comprising a drug identified by the method described above and a pharmaceutically acceptable carrier. As used herein, the term “pharmaceutically acceptable carrier” encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water,

and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. Once the candidate drug has been shown to be adequately bioavailable following a particular route of administration, for example orally or by injection (adequate therapeutic concentrations must be maintained at the site of action for an adequate period to gain the desired therapeutic benefit), and has been shown to be non-toxic and therapeutically effective in appropriate disease models, the drug may be administered to patients by that route of administration determined to make the drug bioavailable, in an appropriate solid or solution formulation, to gain the desired therapeutic benefit. Applicants have identified individual transporter subtype proteins and have described methods for the identification of pharmacological compounds for therapeutic treatments. Pharmacological compounds which are directed against specific transporter subtypes provide effective new therapies with minimal side effects.

This invention provides a method of determining whether a ligand not known to be capable of specifically binding to only one human GABA transporter subtype can specifically bind to only one human GABA transporter subtype which comprises: (a) separately contacting the ligand, under conditions permitting binding to a GABA transporter subtype by a ligand known to bind thereto, with each of (1) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-1 transporter encoded by the plasmid; (2) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-2 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-2 transporter encoded by the plasmid; (3) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-3 transporter encoded by the plasmid; and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-3 transporter encoded by the plasmid; and (4) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human BGT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human BGT-1 transporter encoded by the plasmid; and (b) determining whether the ligand specifically binds to only one of the cell surface human GABA transporter subtypes, but not to the other three, so as to determine whether the ligand is capable of specifically binding to only one human GABA transporter subtype.

This invention further provides a method of determining whether a ligand not known to be capable of specifically binding to only one human GABA transporter subtype can specifically bind to only one human GABA transporter subtype which comprises: (a) separately preparing a cell extract from each of (1) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-1 transporter encoded by the plasmid; (2) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-2 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-2 transporter encoded by the plasmid; (3) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-3 transporter encoded by the plasmid; and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-3 transporter

encoded by the plasmid; and (4) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human BGT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human BGT-1 transporter encoded by the plasmid; (b) separately isolating membrane fractions from each of the cell extracts prepared in step (a); (c) separately incubating the ligand with each of the membrane fractions isolated in step (b) under conditions permitting binding to a human GABA transporter subtype by a ligand known to bind thereto; and (d) determining whether the ligand specifically binds to only one of the membrane-bound human GABA transporter subtypes, but not to the other three, so as to determine whether the ligand is capable of specifically binding to only one human GABA transporter subtype.

This invention provides a method of determining whether a ligand not known to be capable of specifically inhibiting only one human GABA transporter subtype can specifically inhibit only one human GABA transporter subtype which comprises: (a) separately contacting the ligand, under conditions permitting GABA transporter inhibition by a ligand known to inhibit a GABA transporter in the presence of GABA, with each of (1) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-1 transporter encoded by the plasmid; (2) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-2 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-2 transporter encoded by the plasmid; (3) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-3 transporter encoded by the plasmid; and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-3 transporter encoded by the plasmid; and (4) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human BGT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human BGT-1 transporter encoded by the plasmid; and (b) determining whether the ligand specifically inhibits the uptake of GABA for only one of the cell surface human GABA transporter subtypes, but not to the other three, so as to determine whether the ligand is capable of specifically inhibiting only one human GABA transporter subtype.

This invention provides a method of determining whether a ligand not known to specifically inhibit only the human GAT-1 transporter subtype can specifically inhibit only the human GAT-1 transporter subtype which comprises: (a) separately contacting the ligand, under conditions permitting GABA transporter inhibition by a ligand known to inhibit a GABA transporter in the presence of GABA, with each of (1) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-1 transporter encoded by the plasmid; (2) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-2 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human GAT-2 transporter encoded by the plasmid; (3) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human GAT-3 transporter encoded by the plasmid; and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human

GAT-3 transporter encoded by the plasmid; and (4) a mammalian cell (i) comprising a plasmid which comprises DNA encoding a human BGT-1 transporter and which is adapted for expression in a mammalian cell, and (ii) which expresses on its surface the human BGT-1 transporter encoded by the plasmid; and (b) determining whether the ligand specifically inhibits the uptake of GABA for only the cell surface human GAT-1 transporter subtype, but not to the other three, so as to determine whether the ligand is capable of specifically inhibiting only the human GAT-1 transporter subtype. A ligand is determined to specifically inhibit the GAT-1 transporter when the uptake of GABA by the cell is inhibited only in cells expressing the GAT-1 subtype of GABA transporters. GABA uptake can be determined by two methods. First, GABA uptake can be followed using GABA transporter expressing cells in the presence of radiolabeled GABA and then determining the cell associated radioactive counts in the presence or absence of a potential inhibitor under conditions that allow known GABA transport inhibitors to inhibit GABA transport. Secondly, GABA uptake can be followed using GABA transporter expressing cells in the presence of GABA and determining the change in electrical conductance of the GABA expressing cell in the presence and absence of a potential inhibitor under conditions that allow known GABA transporter inhibitors to inhibit GABA transport. Changes in electrical conductance of a GABA transporter in a cell membrane can be measured using standard electrophysiology apparatuses designed for either whole cell electrophysiology or for a fraction of the plasma membrane of the GABA transporter expressing cell (i.e. patchclamping). Conditions for determining the electrophysiology of GABA transporter expressing cells are known in the art (Mager, et al. (1993)).

Elucidation of the molecular structures of the neuronal GABA and taurine transporters is an important step in the understanding of GABAergic neurotransmission. This disclosure reports the isolation, amino acid sequence, and functional expression of a cDNA clones from rat brain which encode a GABA transporters and a cDNA clone from rat brain which encodes a taurine transporter. This disclosure reports the isolation, amino acid sequence, and functional expression of cDNA clones which encode human GABA transporters. The identification of these transporters will play a pivotal role in elucidating the molecular mechanisms underlying GABAergic transmission, and should also aid in the development of novel therapeutic agents. Complementary DNA clones (designated rB14b, K438-rB8b or rB8b, and rB16a) encoding two GABA transporters and a taurine transporter, respectively, have been isolated from rat brain, and their functional properties have been examined in mammalian cells. The clones, K438-rB8b and rB8b have similar pharmacology and as a result the clones were used interchangeably. In the experiments concerning the rat GAT-3 clones, data may have been generated with K438-rB8b or rB8b. When multiple individual experiments were pooled, the individual experiments could have been carried out with either rB8b or K438-rB8b. The nucleotide sequence of rB14b predicts a protein of 602 amino acids, K438-rB8b predicts a protein of 627 amino acids, rB8b predicts a protein of 575 amino acids, and rB16a predicts a protein of 621 amino acids, with 12 highly hydrophobic regions compatible with membrane-spanning domains. When incubated with 50 nM [^{3} H]GABA, COS cells transiently transfected with rB14b or, rB8b or K438-rB8b, accumulated greater than 50-fold as much radioactivity as non-transfected control cells. The transporters encoded by rB14b and rB8b or K438-rB8b display high-affinity for GABA ($K_m=4 \mu M$) and

are dependent on external sodium and chloride. Similarly, when incubated with 50 nM [^{3} H]taurine, Cos cells transiently transfected with rB21a accumulated approximately 7-fold as much radioactivity as non-transfected control cells. The pattern of expression of mRNA encoding two GABA transporters has been examined in the rat brain. Additionally, complementary DNA clones (designated hGAT-3, hHE7a, hS3a) and a genomic DNA clone encoding human GABA transporters have been isolated and their functional properties examined in mammalian cells.

Analysis of the GABA and taurine transporter structure and function provides a model for the development of drugs useful for the treatment of epilepsy, generalized anxiety, migraine, ischemia and other neurological disorders.

This invention identifies for the first time three new mammalian transporter proteins, their amino acid sequences, and their mammalian genes. The invention further identifies the human homologues of two mammalian GABA transporter proteins, their amino acid sequence and their human genes. The information and experimental tools provided by this discovery are useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for these new transporter proteins, their associated mRNA molecules, or their associated genomic DNAs. The information and experimental tools provided by this discovery will be useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for these new transporter proteins, their associated mRNA molecules, or their associated genomic DNAs. This invention discloses for the first time that known anticonvulsant drugs are specific inhibitors of only the GAT-1 transporter. This invention thereby allows for screening of potential anticonvulsant drugs in vitro.

Specifically, this invention relates to the first isolation of three mammalian cDNAs and genomic clones encoding GABA and taurine transporters and the first isolation of cDNAs and a genomic clone encoding the human homologues of two mammalian GABA transporters. The new mammalian genes for these transporters identified herein as rB14b, rB8b or K438-rB8b, and rB16a have been identified and characterized, and a series of related cDNA and genomic clones have been isolated. In addition, the mammalian GABA and mammalian taurine transporters have been expressed in Cos7 cells by transfecting the cells with the plasmids EVJB-rB14b, EVJB-rB8b (truncated clone), and EVJB-rB16a. The pharmacological binding properties of the proteins encoded have been determined, and these binding properties classify these proteins as GABA transporters and a taurine transporter. Mammalian cell lines expressing the mammalian and human GABA transporters and the mammalian taurine transporter on the cell surface have been constructed, thus establishing the first well-defined, cultured cell lines with which to study the GABA and taurine transporters.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

MATERIALS AND METHODS

Materials for Mammalian GABA Transporter Studies:

[^{3} H]GABA ^{3} (98.9 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). GABA, taurine, hypotaurine, poly-D-lysine hydrobromide (average molecular weight, 67,700), ara-C and β -alanine, betaine and L-DABA (L-(2,4) diaminobutyric acid) were from Sigma

Chemical Company (St. Louis, Mo.); guvacine, nipecotic acid, OH-nipecotic (hydroxynipecotic acid), NNC-711, and THPO (4,5,6,7-tetrahydroisoxazolo (4,5-c]pyridin-3-ol) were from RBI (Natick, Mass.). ACHC (cis-3-aminocyclohexanecarboxylic acid) is available from Research Biochemicals International, 1 Strathmore Rd., Natick, Mass., 01760. ($\pm$) Nipecotic acid, guvacine, and NNC-711 were from Research Biochemical Incorporated (Natick, Mass.). CI-966 (Bjorge et al., 1990) and SK&F-89976-A (Ali et al., 1985) were synthesized as described previously. Tiagabine was synthesized as described by Andersen et al. (1993).

Materials for Mammalian Taurine Transporter Studies:

[^{3} H]taurine (25.6 Ci/mmol) was from New England Nuclear (Boston, Mass.); taurine, GABA ^{2} , hypotaurine, AEPA, AMSA, APSA, CSA, MEA, and β -alanine were from Sigma Chemical Corporation (St. Louis, Mo.); GES can be prepared according to the method of Fujii, et al. (1975) (117). Cell culture media were obtained from Specialty Media (Lavallete, N.J.), and serum was from UBI (Lake Placid, N.Y.). Cell cultures were maintained at 37° C. in a humidified 95% air/5% CO $_2$ atmosphere.

Cloning and Sequencing of Mammalian GABA Transporters:

A rat brain cDNA library in the Lambda ZAP II vector (Stratagene, La Jolla, Calif.) was screened at reduced stringency using probes representing the complete coding region of the rat GABA transporter cDNA (GAT-1 (21)). Exact primers derived from the nucleotide sequence of GAT-1 were used to generate GAT-1 PCR products from randomly-primed rat brain cDNA; the GAT-1 probes were then labeled and used to screen the library under reduced stringency as previously described (98). Lambda phage hybridizing with the probes at low stringency were plaque purified and rescreened at high stringency to eliminate clones which were identical to GAT-1. One of the clones hybridizing at high stringency was subsequently confirmed by sequence analysis to encode GAT-1 (31). Clones hybridizing only at low stringency were converted to phagemids by in vivo excision with f1 helper phage. Nucleotide sequences of double-stranded cDNAs in pbluescript were analyzed by the Sanger dideoxy nucleotide chain-termination method (89) using Sequence (U.S. Biochemical Corp., Cleveland, Ohio).

Expression of Mammalian GABA Transporters:

cDNA clones (designated rB14b and rB8b) representing coding regions of two putative transporters were cloned into the eukaryotic expression vector pEVJB (modified from pcEXV-3; (70)). Utilizing restriction enzyme sites present in pbluescript, rB14b was subcloned as a 2.0 kb HindIII/XbaI fragment which contained 126 base pairs of 5'-untranslated sequence and 94 base pairs of 3'-untranslated sequence. Similarly, rB8b was subcloned as a 2.1 kb XbaI/SalI fragment containing 0.3 kb of 3'-untranslated sequence. Transient transfections of COS cells were carried out using DEAE-dextran with DMSO according to the method of Lopata et al. (58) with minor modifications. COS cells were grown (37° C., 5% CO $_2$) in high glucose Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum, 100 U/ml penicillin G, and 100 μ g/ml streptomycin sulfate. Cells were routinely used two days after transfection for transport studies.

Transport Studies of Mammalian GABA Transporters:

To measure transport, COS cells grown in 6-well (well diameter=35 mm) or 24-well (well diameter=18 mm) plates were washed 3 $\times$ with HEPES-buffered saline (HBS, in mM: NaCl, 150; HEPES, 20; CaCl $_2$, 1; glucose, 10; KCl, 5; MgCl $_2$, 1; pH 7.4) and allowed to equilibrate in a 37° C.

water bath. After 10 minutes the medium was removed and a solution containing [^{3} H]GABA (New England Nuclear, sp. activity=89.8 Ci/mmol) and required drugs in HBS was added (1.5 ml/35 mm well; 0.5 ml/18 mm well). Non-specific uptake was defined in parallel wells with 1 mM unlabeled substrate, and was subtracted from total uptake (no competitor) to yield specific uptake; all data represent specific uptake. Plates were incubated at 37° C. for 10 minutes unless indicated otherwise, then washed rapidly 3 $\times$ with ice-cold HBS. Cells were solubilized with 0.05% sodium deoxycholate/0.1N NaOH, an aliquot neutralized with 1N HCl, and radioactivity was determined by scintillation counting. Protein was quantified in an aliquot of the solubilized cells using a BIO-RAD protein assay kit, according to the manufacturers directions.

Northern Blot Analysis of RNA Encoding Mammalian Transporters:

Total cellular RNA was isolated from rat brain and liver using RNazol (Cinna/Biotech Laboratories Inc.; Houston, Tex.) as outlined by the manufacturer. Denatured RNA samples (25 μ g) were separated in a 1.0% agarose gel containing 3.3% formaldehyde. RNAs were transferred to nylon membranes (Genescreen Plus; New England Nuclear, Boston, Mass.) by overnight capillary blotting in 10 $\times$ SSC. Northern blots were rinsed and then baked for 2 hours at 80° C. under vacuum. Prehybridization was for 2 hours at 65° C. in a solution containing 50% formamide, 1M NaCl, 10% dextran sulfate, and 1% sodium dodecyl sulfate. Blots were hybridized overnight at 65° C. with ^{32} P-labeled DNA probes (randomly primed GAT-2 full-length or GAT-3 truncated or full-length cDNA clones) in prehybridization mixture containing 100 μ g/ml sonicated salmon sperm DNA. The blots were washed successively in 2 $\times$ SSC/2% SDS, 1 $\times$ SSC/2% SDS, and 0.2 $\times$ SSC/2% SDS at 65° C., then exposed to Kodak XAR-5 film with one intensifying screen at -90° C. for four days.

Tissue Localization Studies:

To identify tissues expressing mRNAs for the novel GABA transporters and the previously cloned GABA transporter GAT-1 (31), specific PCR primers (25 mers) were designed such that $\approx$ 700 base pair fragments encoding TMs 1 through 5 of each transporter could be amplified and detected by hybridization with ^{32} P-labeled oligonucleotides. For rB14b, the sequences of the sense and anti-sense oligonucleotides were derived from amino acids 36 to 43 (5'-GACCAACAAGATGGAGTTCGTA CTG) (Seq. I.D. No. 14) and 247 to 254 (5'-TGTTACTCCTCGGATCAACAGGACC) (Seq. I.D. No. 15); for K438-rB8b, the oligonucleotides were derived from amino acids 52 to 60 (5'-GGAGTTCGTGTTGAGCGTAGGAGAG) (Seq. I.D. No. 16) and 271 to 279 (5'-GAAC TTGATGCCTTCCGAGGCACCC) (Seq. I.D. No. 17); and for GAT-1 (31), the oligonucleotide sequences were derived from amino acids 50 to 57 (5'-ACGCTTCGACTTCCTCATGTCCTGT) (Seq. I.D. No. 18) and 274 to 282 (5'-GAATCAGACAGCTTTCGGAAGTTGG) (Seq. I.D. No. 19). Primers were also designed to amplify the cDNA encoding cyclophilin, a constitutively expressed gene, as a control (5'-GTCTGCTTCGAGCTGTTTGCAGACA, sense (Seq. I.D. No. 20); 5'-TTAGAGTTGTCCACAGTCGGAGATG, anti-sense (Seq. I.D. No. 21)) (21). To detect amplified sequences, oligonucleotide probes were synthesized for GAT-1, rB14b, and K438-rB8b which corresponded to amino acids 196 to 219, 161 to 183, and 207 to 229, respectively. Each probe

was shown to hybridize with its respective transporter cDNA and not with any other transporter cDNA under study.

Poly A⁺ RNA (1 μ g, Clontech, Palo Alto, Calif.) from each of seven rat tissues was converted to single-stranded cDNA by random priming using Superscript reverse transcriptase (BRL, Gaithersburg, Md.). PCR reactions were carried out in a buffer containing 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 mM dNTP's, 1 μ M each primer, and Taq polymerase with either cDNA, RNA, water, or a control plasmid for 30 cycles of 94° C./2 min., 68° C./2 min., 72° C./3 min. PCR products were separated by electrophoresis in 1.2% agarose gels, blotted to nylon membranes (Genescreen Plus; New England Nuclear, Boston, Mass.), and hybridized at 40° C. overnight with ³²P-labeled oligonucleotide probes in a solution containing 50% formamide, 10% dextran sulfate, 5×SSC, 1×Denhardt's, and 100 μ g/ml sonicated salmon sperm DNA. Blots were washed successively in 2×SSC 20 at room temperature and 0.1×SSC at 50° C., and exposed to Kodak XAR film for 0.5 to 4 hours with an intensifying screen at -70° C.

Cloning and Sequencing of Mammalian Taurine Receptor:

A rat brain cDNA library in the Lambda ZAP II vector (Stratagene, La Jolla, Calif.) was screened at low stringency with the complete coding region of the rat GABA transporter cDNA (GAT-1; (31)). Exact primers were used to generate PCR products from randomly-primed rat brain cDNA; the products were labeled and used to screen the library under reduced stringency (25% formamide, 40° C. hybridization; 0.1×SSC, 40° C. wash) as previously described (98). Lambda phage hybridizing at low stringency with the GAT-1 sequence were plaque purified and rescreened with the same probes at high stringency (50% formamide, 40° C. hybridization; 0.1×SSC, 50° C. wash) to eliminate clones identical to GAT-1. Clones hybridizing only at low stringency were converted to phagemids by in vivo excision with f1 helper phage. Nucleotide sequences of double-stranded cDNAs in pBluescript were analyzed by the Sanger dideoxy nucleotide chain-termination method (89) using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio).

Expression of Mammalian Taurine Transporter:

A complementary DNA (designated rB16a) containing the complete coding region of a putative transporter was cloned into the eukaryotic expression vector pEVJB (modified from pcEXV-3; (70)) as a 2.5 kb XbaI/SalI fragment using restriction enzyme sites within the vector. In addition to the coding region, 0.1 kb of 5'-untranslated sequence and 0.5 kb of 3'-untranslated sequence were included in the construct. Transient transfections of COS cells with the plasmid pEVJB-rB16a were carried out using DEAE-dextran with DMSO according to the method of Lopata et al. (58) with minor modifications. COS cells were grown (37° C., 5% CO) in high glucose Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum, 100 U/ml penicillin G, and 100 μ g/ml streptomycin sulfate. Cells were routinely used two days after transfection for transport studies.

Transport Studies of Mammalian Taurine Transporter:

To measure transport, COS cells grown in 6-well (well diameter=35 mm) or 24-well (well diameter=18 mm) plates were washed 3× with HEPES-buffered saline (HBS, in mM: NaCl, 150; HEPES, 20; CaCl₂, 1; glucose, 10; KCl, 5; MgCl₂, 1; pH 7.4) and allowed to equilibrate in a 37° C. water bath. After 10 minutes the medium was removed and a solution containing [³H]taurine (New England Nuclear, sp. activity=25.6 Ci/mmol) and required drugs in HBS was added (1.5 ml/35 mm well; 0.5 ml/18 mm well). Non-

specific uptake was defined in parallel wells with 1 mM unlabeled taurine and was subtracted from total uptake (no competitor) to yield specific uptake; all data represent specific uptake. Plates were incubated at 37° C. for 10 minutes unless indicated otherwise, then washed rapidly 3× with ice cold HBS. Cells were solubilized with 0.05% sodium deoxycholate/0.1N NaOH, an aliquot was neutralized with 1N HCl, and radioactivity was determined by scintillation counting. Protein was quantified in an aliquot of the solubilized cells using a BIO-PAD protein assay kit, according to the manufacturer's directions.

PCR Tissue Localization Studies of Mammalian Taurine Transporter:

To identify tissues expressing mRNA for the taurine transporter, exact primers (25 mers) were designed such that a 707 base pair fragment of rB16a could be amplified from cDNA and detected by Southern blot analysis. The sequences of the sense and anti-sense primers were derived from amino acids 40 to 47 (5'-TCAGAGGGAGAAGTGGTCCAGCAAG) (Seq. I.D. No. 22) and 268 to 275 (5'-ATTTCATGCCTTCACCAGCACCTGG) (Seq. I.D. No. 23), respectively. Primers were also designed to amplify the cDNA encoding cyclophilin (12), a constitutively expressed gene, as a control (5'-ACGCTTCGACTTCCTCATGTCCTGT, sense (Seq. I.D. No. 24); 5'-TTAGAGTTGTCCACAGTCGGAGATG, antisense) (Seq. I.D. No. 21). To detect amplified sequences, an oligonucleotide probe was synthesized (corresponding to amino acids 249 to 271) which was specific for rB16a. Poly A⁺ PNA (1 μ g, Clontech, Palo Alto, Calif.) from each of seven rat tissues was converted to single-stranded cDNA by random priming using Superscript reverse transcriptase (BRL, Gaithersburg, Md.). PCR reactions were carried out in a buffer containing 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 mM dNTP's, 1 μ M each primer, Taq polymerase, and either cDNA, RNA, water, or a control plasmid containing rB16a for 30 cycles of 94° C./2 min., 68° C./2 min., 72° C./3 min. PCR products were separated by electrophoresis in 1.2% agarose gels, blotted to nylon membranes (Genescreen Plus; New England Nuclear, Boston, Mass.), and hybridized at 40° C. overnight with specific ³²P-labeled oligonucleotides in a solution containing 50% formamide, 10% dextran sulfate, 5×SSC, 1×Denhardt's, and 100 μ g/ml of sonicated salmon sperm DNA. Blots were washed at high-stringency (0.1×SSC, 50° C.) and exposed to Kodak XAR film for 0.5 to 4 hours with one intensifying screen at -70° C.

Northern Blot Analysis of mRNA Encoding Mammalian Taurine Transporter:

Samples of poly A⁺ RNA isolated from each of eight rat tissues (5 μ g, Clontech; Palo Alto, Calif.) were separated in a 1.0% agarose gel containing 3.3% formaldehyde and transferred to a nylon membrane (Genescreen Plus; New England Nuclear, Boston, Mass.) by overnight capillary blotting in 10×SSC. Prior to hybridization, the Northern blot was incubated for 2 hours at 42° C. in a solution containing 50% formamide, 1M NaCl, 10% dextran sulfate, and 1% sodium dodecyl sulfate (SDS). The blot was hybridized overnight at 42° C. with ³²P-labeled DNA probe (randomly-primed HindIII/KpnI fragment of rB16a representing amino acids 6-336) in the prehybridization solution containing 100 μ g/ml sonicated salmon sperm DNA. The blot was washed successively in 2×SSC/2% SDS, 1×SSC/2% SDS, and 0.2×SSC/2% SDS at 65° C. and exposed to Kodak XAR-5 film with one intensifying screen at -70° C. for 1-4 days. To confirm that equal amounts of RNA were present in each

lane, the same blot was rehybridized with a probe encoding cyclophilin (12).

Use of PCR to Identify Human cDNA Libraries for Screening:

For hGAT-2, the sequences of the rat PCP primers were 5'-GACCAACAAGATGGAGTT (sense) (Seq. I.D. No. 25) and 5'-TGTTACTCCTCGGATCAA (antisense) (Seq. I.D. No. 26). PCR reactions were carried out in a buffer containing 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 mM dNTP's, 1 μM each primer, Taq polymerase, and an aliquot of a lambda phage library, water, or a control plasmid for 40 cycles of 94° C. for 2 min., 50° C. for 2 min., and 72° C. for 3 min. For hGAT-3, the sequences of the degenerate primers were 5'-TGGAATTCG (G/C)CAA(C/T)GTITGG(C/A)GITT(C/T)CCITA (sense) (Seq. I.D. No. 27) and 5'-TCGCGGCCGCAA(A/G)AAGATCTGIGTIGCIGC(A/G)TC (antisense) (Seq. I.D. No. 28). PCR reactions were carried out as described above for 40 cycles of 94° C. for 2 min., 40° C. for 2 min., and 72° C. for 3 min. PCR products were separated by electrophoresis in 1.2% agarose gels, blotted to nylon membranes (Genescreen Plus; New England Nuclear, Boston, Mass.), and hybridized at 40° C. overnight with ³²P-labeled probes in a solution containing 25% formamide, 10% dextran sulfate, 5×SSC, 1×Denhardt's, and 100 μg/ml of sonicated salmon sperm DNA. Blots were washed at low stringency (0.1×SSC, 40° C.) and exposed to Kodak XAR film for up to three days with one intensifying screen at -70° C.

Isolation and Sequencing of Human Clones:

Human cDNA libraries in the Lambda ZAP II vector (Stratagene, La Jolla, Calif.) that were identified as containing hGAT-2 or hGAT-3 were screened under either reduced stringency (25% formamide, 40° C. hybridization; 0.1×SSC, 40° C. wash) or high stringency (50% formamide, 40° C. hybridization; 0.1×SSC, 50° C. wash). Hybridizing lambda phage were plaque purified and converted to phagemids by in vivo excision with f1 helper phage. Nucleotide sequences of double-stranded cDNAs in pbluescript were analyzed by the Sanger dideoxy nucleotide chain-termination method (89) using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Fragments of genomic clones in the lambda FIX II vector were subcloned into pUC18 prior to double-stranded sequencing.

Preparation of Primary Brain Cell Cultures:

Astrocytes, neurons and meningeal fibroblasts were prepared from the brains of E19 embryonic rats. Briefly, the brains were removed, dissected free of meninges, and trypsinized. Cells were dissociated mechanically by passage through a Pasteur pipette, and resuspended in DMEM containing 10% fetal bovine serum and antibiotics. The cells were added to tissue culture dishes that had been previously coated with 10 μM poly-D-lysine.

For astrocytes, the cells were plated at a density of approximately 3×10⁶ cells per 100 mm dish. The astrocytes were allowed to reach confluence, then passaged 1 or 2 times prior to harvesting. For neurons, a plating density of 15×10⁶ Cells per 100 mm dish was employed; the medium was supplemented with insulin. Cytosine arabinoside (ara-C) was added to a final concentration of 10 μM on day 2 or 3 to inhibit the proliferation of non-neuronal cells. The neurons were harvested 1 week after plating. To obtain meningeal fibroblasts the meninges were trypsinized, then mechanically dissociated as described above. The cells recovered from a single embryo were plated into a 100 mm dish, grown to confluence, and passaged 1-2 times prior to harvesting.

Isolation of RNA from Cell Cultures:

Plates were placed on ice and quickly rinsed twice with ice-cold phosphate-buffered saline (PBS). Cells were then dissolved in 10 ml lysis solution (7M urea, 350 mM NaCl, 2% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0) and transferred to a sterile tube. Lysates were homogenized (Virtis, lowest speed, 5 seconds) and then digested with proteinase K (0.1 mg/ml) at 37° C. for 30 minutes. Samples were extracted twice with phenol/chloroform and once with chloroform before ethanol precipitation. Total RNA was collected by centrifugation, resuspended in diethylpyrocarbonate (DEPC)-treated water, and stored at -20° C. until use.

Detection of Transporter mRNAs Using PCR:

To identify cell types expressing mRNAs for the GABA transporters GAT-1, GAT-2, and GAT-3, specific PCR primers (25 mers) were designed such that ≈700 base pair fragments encoding transmembrane domains 1 through 5 of each transporter could be amplified and detected by hybridization with ³²P-labeled oligonucleotides. For rB14b (GAT-2), the sequences of the sense and anti-sense oligonucleotides were derived from amino acids 36 to 43 (5'-GACCAACAAGATGGAGTTCGTACTG) (Seq. I.D. No. 14) and 247 to 254 (5'-TGTTACTCCTCGGATCAACAGGACC) (Seq. I.D. No. 15); for K438-rB8b (GAT-3), the oligonucleotides were derived from amino acids 52 to 60 (5'-GGAGTTCGTGTTGAGCGTAGGAGAG) (Seq. I.D. No. 16) and 271 to 279 (5'-GAACCTTGATGCCTTCCGAGGCACCC) (Seq. I.D. No. 17). and for GAT-1 (31), the oligonucleotide sequences were derived from amino acids 50 to 57 (5'-ACGCTTCGACTTCCTCATGTCCTGT) (Seq. I.D. No. 18) and 274 to 282 (5'-GAATCAGACAGCTTTCGGAAGTTGG) (Seq. I.D. No. 19). To detect amplified sequences, oligonucleotide probes were synthesized for GAT-1, GAT-2, and GAT-3 which corresponded to amino acids 196 to 219, 161 to 183, and 207 to 229, respectively. Each probe was shown to hybridize with its respective transporter cDNA and not with the other transporter cDNAs.

Total RNA (0.5 μg) isolated from cultured neurons, astrocytes, and fibroblasts was converted to single-stranded cDNA by random priming using Superscript reverse transcriptase (BRL, Gaithersburg, Md.). PCR reactions were carried out in a buffer containing 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 mM dNTP's, 1 μM each primer, and Taq polymerase with either cDNA, RNA, water, or a control plasmid for 30 cycles of 94° C./2 min., 68° C./2 min., 72° C./3 min. PCR products were separated by electrophoresis in 1.2% agarose gels, blotted to nylon membranes (Genescreen Plus; New England Nuclear, Boston, Mass.), and hybridized at 40° C. overnight with ³²P-labeled oligonucleotide probes in a solution containing 50% formamide, 10% dextran sulfate, 5×SSC, 1×Denhardt's, and 100 μg/ml sonicated salmon sperm DNA. Blots were washed successively in 2×SSC, 0.1% SDS at room temperature and 0.1×SSC, 0.1% SDS at 50° C., and exposed to Kodak XAR film for 0.5 to 4 hours with an intensifying screen at -70° C.

In Situ Hybridization:

Male Sprague-Dawley rats (Charles River) were decapitated and the brains rapidly frozen in isopentane. Sections were cut on a cryostat, thaw-mounted onto poly-L-lysine coated coverslips, and stored at -80° C. until use. Tissue was fixed in 4% paraformaldehyde, treated with 5 mM dithiothreitol (DTT), acetylated (0.25% acetic anhydride in 0.1M

triethanolamine), and dehydrated. Tissue was prehybridized (1 hour, 40° C.) in a solution containing 50% formamide, 4×SSC (0.6M NaCl/0.06M sodium citrate), 1×Denhardt's solution (0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin), 50 mM DTT, 500 µg/ml salmon sperm DNA, 500 µg/ml yeast tRNA, 10% dextran sulfate, then hybridized overnight with ³⁵S-labeled anti-sense oligonucleotides (45 mers) in the same solution. After washing and dehydration, sections were apposed to Kodak X-OMAT AR film for 4 days at -20° C. To verify the specificity of the hybridization signal, parallel tissues were pretreated with 100 µg/ml RNase A (37°, 30 minutes) prior to hybridization. Two different oligonucleotides designed to separate regions of the GABA transporters (loop region between transmembrane domains III and IV, 3'untranslated region) showed identical patterns of hybridization.

LIPOPHILIC GABA TRANSPORT INHIBITORS SK&F 89976-A, TIAGABINE, CI-966, AND NNC-711 ARE SELECTIVE FOR THE CLONED GABA TRANSPORTER GAT-1

Stable Transfection:

Stable cell lines for human GAT-1, rat GAT-2, human GAT-3, and human BGT-1 were generated in LM(tk⁻) cells using the calcium phosphate method and selection in G-418, as described previously (Weinshank et al., 1992). Cells were grown under standard conditions (37° C., 5% CO₂) in Dulbecco's modified Eagles's medium (GIBCO, Grand Island, N.Y.). Human GAT-1 is the human homologue of the rat transporter GAT-1 (Nelson et al., 1990) which we recloned. Human GAT-3 and human BGT-1 are the human homologues of rat GAT-3 (Borden et al., 1992) and dog BGT-1 (Yamauchi et al., 1992); a more complete description of these homologues will be described in subsequent communications (Borden et al., 1994).

Transient Transfection:

GABA transport by rat GAT-1 and rat GAT-3 was examined in transiently transfected cells. Transient transfections were conducted as previously described (Smith et al., 1992), with the following modifications: COS cells grown in 75 cm² or 150 cm³ flasks in DMEM with 10% bovine calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate (37° C., 5% CO₂) were transfected using DEAE-dextran. On the day following transfection the cells were split into 24-well assay plates (well diameter=18 mm) coated with poly-D-lysine (10 µg/ml), and transport was measured 24 hours later.

Transport Assay:

Transport by attached cells was measured as described previously (Borden et al., 1992), with the following modifications. Cells grown in 24-well plates were washed 3× with HEPES-buffered saline (HBS, in mM: NaCl, 150; HEPES, 20; CaCl₂, 1; glucose, 10; KCl, 5; MgCl₂, 1; pH 7.4) and allowed to equilibrate on a 37° C. slide warmer. After 10 minutes the medium was removed and unlabeled drugs in HBS were added (450 µl/well). Transport was initiated by adding 50 µl per well of a concentrated solution of [³H] GABA in HBS (final concentration=50 nM). Non-specific uptake was defined in parallel wells with 1 mM unlabeled GABA, and was subtracted from total uptake (no competitor) to yield specific uptake; all data represent specific uptake. Plates were incubated at 37° C. for 10 minutes, then washed rapidly 3× with ice-cold HBS, using a 24-position plate washer (Brandel, Gaithersburg, Mass.; model PW-12). Cells were solubilized with 0.05% sodium deoxycholate/0.1N NaOH (0.25 ml/well), an aliquot neutralized with 1N HCl, and radioactivity was determined by scintillation counting. Protein was quantified in an aliquot of

the solubilized cells using a BIO-RAD protein assay kit, according to the manufacturer's directions.

Lipophilic inhibitors were dissolved in DMSO. The final concentration of DMSO in the transport assay was ≤2%, and control experiments demonstrated that this concentration had no significant effect on transport.

Data Analysis:

Competition curves were conducted in duplicate, using 10 concentrations of unlabeled drug. IC₅₀ values (concentrations resulting in 50% inhibition of uptake) were derived using software from GraphPad Inplot (San Diego, Calif.). Data were not corrected for the concentration of radioligand since [³H]GABA was employed at a concentration well below its K_M; however, since initial rates were not studied, data are presented as IC₅₀. All data throughout this invention represent means±SEM.

GABA TRANSPORT IN NEURONAL AND GLIAL CELL CULTURES: CORRELATION OF PHARMACOLOGY AND mRNA LOCALIZATION

Preparation of Primary Rat Astrocyte Cell Cultures:

Primary glial cultures were established from newborn Sprague-Dawley rat brains. Brains minus cerebella were removed aseptically and carefully dissected free of meninges. A single-cell suspension was made by mincing the brains, then incubating them with 0.25% trypsin/1 mM EDTA for 2–3 minutes. Following inactivation of the trypsin by addition of complete medium (DMEM containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate), the cells were allowed to settle and the supernatant decanted; this procedure was then repeated. The cells were dissociated by trituration through a serological pipette. Separate procedures were then used for the preparation of Type-1 and O-2A/Type-2 astrocyte cultures, as will now be described.

For the preparation of Type-1 astrocyte cultures, cells were plated onto uncoated 75 cm² flasks at low density (1 brain per 10 flasks, or approximately 8×10⁴ cells/cm²). The medium was changed the following day and twice a week thereafter. After 2–3 weeks the Type 1 astrocytes formed a confluent monolayer, with only minimal contamination of O-2A/Type-2 cells. The cells were then split into 24-well plates (typically, each 75 cm² flask was split into six 24-well plates) and 1–2 weeks later, transport was measured as described above. Such cultures stained heavily with an anti-GFAP antibody (not shown).

Cultures highly enriched in O-2A/Type-2 cells were prepared from mixed astrocyte cultures which consisted of a monolayer of Type 1 cells upon which rested large numbers of O-2A/Type 2 cells. It had previously been shown that O-2A/Type 2 cells could be purified from such cultures by overnight shaking, which releases the O-2A/Type 2 cells into the medium (McCarthy and DeVellis, 1980). However, we found this method to be impractical for the large numbers of cells required for the present study, and thus sought a more rapid method of isolation. The same brain cell suspensions as described above were plated at high density (1 brain per 75 cm² flask, or approximately 8×10⁵ cells/cm²) into flasks coated with poly-D-lysine (10 µg/ml). After 24 hours the medium was replaced and the cultures were subsequently fed twice a week. After 2–3 weeks the cultures consisted of a large number of O-2A/Type-2 cells lying on a monolayer of Type-1 astrocytes. The mixed Type-1 and O-2A/Type-2 astrocyte cultures were washed with PBS, then incubated with trypsin/EDTA for 20–30 seconds with vigorous "smacking"; this technique selectively detached the O-2A/Type-2 cells. Complete medium was added to the

flasks and the cell suspension was removed gently, care being taken to not disrupt the underlying Type 1 astrocyte monolayer. The cells were pelleted at low speed, resuspended in complete medium, and added to 24-well plates coated with poly-D-lysine (10 $\mu\text{g}/\text{ml}$). Typically, the cells collected from eight 75 cm^2 flasks were added to twelve 24-well plates; increasing the cell density resulted in greater contamination of Type-1 astrocytes. The cells were used for transport assays two days later, by which time about 85% of the cells had extended processes with the typical morphology of O-2A/Type-2 astrocytes; such cells displayed varying intensities of staining with an anti-GFAP antibody (not shown), similar to previous results (Lillien and Raff, 1990). No attempt was made to distinguish or separate O-2A precursors from the more differentiated Type 2 astrocytes. Preparation of Primary Rat Neuronal Cell Cultures:

Primary neuronal cultures were prepared as described previously (Vaysse et al., 1990). Brains were removed aseptically from E17 embryonic Sprague-Dawley rats brains, carefully dissected free of meninges, and kept on ice. A single-cell suspension was made by mincing the brains, followed by trituration through a serological pipette. The large clumps were allowed to settle, and the supernatant diluted with medium (DMEM/F-12 (1:1) containing 10% fetal calf serum, 100 U/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate, 10 $\mu\text{g}/\text{ml}$ insulin (porcine or bovine), supplemented with 45 mM KCl), and added to 24-well plates (coated with 10 $\mu\text{g}/\text{ml}$ poly-D-lysine) at a density of approximately 2×10^5 cells/ cm^2 . On day three cytosine β -D-arabinofuransoside (3 μM) was added to control the proliferation of non-neuronal cells. The cultures were used for transport assay approximately one week after plating.

Transient Transfection:

Transient transfection was carried out in COS cells as previously described (Smith et al., 1992), with the following modifications. COS cells grown in 75 cm^2 or 150 cm^2 flasks in DMEM with 10% bovine calf serum, 100 U/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate (37° C., 5% CO_2) were transfected using DEAE-dextran. On the day following transfection the cells were split into 24-well assay plates coated with poly-D-lysine (10 $\mu\text{g}/\text{ml}$), and transport was measured 24 hours later.

During the course of this work we noted that compounds typically displayed higher affinity in stable transfections in LM(tk⁻) cell hosts than in transient transfections in COS cell hosts. The difference in affinity was typically 3–4-fold, though this varied from clone to clone. Importantly, expression levels did not appear to alter the order of potencies. This phenomenon should be considered when comparing data using different expression systems.

Transport in Attached Cells:

GABA transport by attached cells was measured as described previously (Borden et al., 1992), with the following modifications. Cells grown in 24-well plates (well diameter=18 mm) were washed 3× with HEPES-buffered saline (HBS, in mM: NaCl, 150; HEPES, 20; CaCl_2 , 1; glucose, 10; KCl, 5; MgCl_2 , 1; pH 7.4) and allowed to equilibrate on a 37° C. slide warmer. After 10 minutes the medium was removed and unlabeled drugs in HBS were added (450 $\mu\text{l}/\text{well}$). Transport was initiated by adding 50 μl per well of a concentrated solution of [³H]GABA in HBS (final concentration=50 nM). Non-specific uptake was defined in parallel wells with 1 unlabeled GABA (10 mM for Type 1 astrocytes), and was subtracted from total uptake (no competitor) to yield specific uptake; all data represent specific uptake. Plates were incubated at 37° C. for 10 minutes, then washed rapidly 3× with ice-cold HBS, using a

24-position plate washer (Brandel, Gaithersburg, Mass.; model PW-12). Cells were solubilized with 0.05% sodium deoxycholate/0.1N NaOH (0.25 ml/well), an aliquot neutralized with 1N HCl, and radioactivity was determined by scintillation counting. Protein was quantified in an aliquot of the solubilized cells using a BIO-RAD protein assay kit, according to the manufacturer's directions.

Brain Aggregate Preparation:

Brain aggregates were prepared as described in Forray and El-Fakahany (1990), with minor modifications. Briefly, adult rat brains were removed from the skull and the cerebral cortex was dissected and placed in ice-cold HBS. The tissue was forced through a nylon mesh bag (200 μm pore; Nitex) followed by passage through a second nylon bag (130 μm pore size; Nitex). The preparation was washed once by centrifugation, then resuspended in HBS.

Transport by Brain Aggregates:

GABA transport by brain aggregates was determined in suspension in 96-well microtiter plates (total incubation volume=250 μl), using an assay similar to that employed for membrane receptor binding (see Zgombick et al., 1991). Briefly, [³H]GABA (final concentration=50 nM) and unlabeled drugs were added to 96-well plates (Corning), and transport was initiated by the addition of brain aggregates. Non-specific uptake was determined with 1 mM unlabeled GABA. The reaction was continued for 10 minutes (37° C.), then terminated by rapid filtration through pre-soaked (0.5% polyethyleneimine) GF/B glass fiber filters, using a Brandel48R cell harvester. The filters were washed with ice-cold HBS, punched into vials, and radioactivity determined by scintillation counting. Protein was determined in an aliquot of the aggregate preparation using the BIO-RAD protein assay kit.

Northern Blot Analysis of Transporter mRNAs:

Total cellular RNA was isolated from cultured cells using a modification of the method of Chirgwin et al. (1979). Briefly, cells were rinsed with ice-cold phosphate-buffered saline and lysed in guanidine isothiocyanate (5–10 mls/100 mm plate). Lysates were drawn twice through a 20-gauge needle to shear genomic DNA, and RNA was collected by centrifugation through 5.7M cesium chloride (MacDonald et al., 1987). Rat brain poly A⁺ RNA was purchased from Clontech (Palo Alto, Calif.). Denatured RNA samples (5–25 μg) were separated by electrophoresis in 1.0% agarose gels containing 2.7% formaldehyde. RNAs were transferred to GeneScreen Plus nylon membranes (Dupont-NEN, Boston, Mass.) by overnight capillary blotting in 10×SSC. Northern blots were rinsed and then baked for 2 hours at 80° C. under vacuum. Prehybridization was for 1–2 hours at 42° C. in a solution containing 50% formamide, 1M sodium chloride, 10% dextran sulfate and 1.0% SDS. Blots were hybridized overnight at 42° C. with ³²P-labeled randomly primed probes (Prime-It II, Stratagene, La Jolla, Calif.) in prehybridization mixture containing 100 $\mu\text{g}/\text{ml}$ sonicated salmon sperm DNA. The blots were washed successively in 2×SSC/2% SDS, 1×SSC/2% SDS, and 0.2×SSC/2% SDS at 65° C., then exposed to Kodak XAR-5 film with one intensifying screen at –80° C. for up to one week.

To facilitate quantitative comparisons of signal intensities between transporters, cDNA fragments of the same size (≈ 1.5 kb) were prepared as templates for random prime labeling of GAT-1, -GAT-2, GAT-3, and TAUT cDNAs, and the specific activity of each probe was determined by TCA precipitation (Sambrook et al., 1989) 20 prior to hybridization. Specific activities ranged from $1.5\text{--}2.5 \times 10^9$ dpm/ μg DNA for all probes. Since the cDNA encoding the rat betaine/GABA transporter was not available, an ≈ 450 bp

fragment was generated by PCR (see below), subcloned, sequenced, radiolabeled, and used for hybridization of Northern blots as described. Since this fragment of rBGT-1 is much smaller than the other probes, levels of rBGT-1 transcripts cannot be visually compared with those of GAT-1, GAT-2, GAT-3, and TAUT.

After hybridization with transporter cDNAs, all blots were reprobbed for the constitutive mRNA encoding cyclophilin (IB15; Danielson et al., 1988) to confirm that equal amounts of RNA were present in each lane.

Cloning a Fragment of the Rat BGT-1:

Primers based on the nucleotide sequence of the mouse betaine/GABA transporter (Lopez-Corcuera et al., 1992; termed "GAT2" by authors) were used to amplify a fragment of rBGT-1 from rat brain cDNA using PCR. The primers represent nucleotides 268–296 (sense) and 715–740 (antisense) of the mouse sequence (Lopez-Corcuera et al., 1992). First strand cDNA was synthesized from rat brain poly A+ RNA using Superscript reverse transcriptase (BRL, Gaithersburg, Md.). PCR amplification was carried out in a buffer containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each dNTP, 1 uM each primer, Taq polymerase, and either first strand cDNA, RNA, or water for 30 cycles of 94° C./2 min, 60° C./2 min, 72° C./3 min. The ≈450 bp PCR product was separated by agarose gel electrophoresis, isolated on DE81 paper and ligated into pUC18. A portion of the reaction was blotted to a nylon membrane (Zeta-Probe GT, Bio-Rad Laboratories, Richmond, Calif.) and hybridized as described below with a ³²P-labeled oligonucleotide (45 mer) representing the mouse BGT-1 (Lopez-Corcuera et al., 1992) to confirm its identity. Sequence analysis of the subcloned fragment confirmed that it encoded a portion of the rBGT-1; the fragment exhibited 85–94% nucleotide identity and 87–96% amino acid identity with dog, human, and mouse BGT-1 cDNAs, but only 72% nucleotide identity (68% amino acid identity) with the next closest relative, rat GAT-2. This fragment was subsequently reisolated from a single transformant and used in northern blot hybridizations of rBGT-1 mRNA.

Quantitation of Northern Blots:

Autoradiograms were scanned using a Color One Scanner (Apple) and analyzed on an Apple Macintosh computer using Image 1.43 software (NIH). Band intensities were corrected for the amount of mRNA as determined by hybridization to the constitutive mRNA encoding cyclophilin, then normalized to the amount of GAT-1 present in neuronal cultures (for neurons and astrocyte cultures), or the amount of GAT-1 present in brain.

Data Analysis:

Competition curves were conducted in duplicate, using 10 concentrations of unlabeled drug. Curves were analyzed using software from GraphPad Inplot (San Diego, Calif.). Data were not corrected for the concentration of radioligand since [³H]GABA was employed at a concentration well below its K_M; however, since initial rates were not studied, data are presented as IC₅₀. Data represent means±SEM.

1. GABA Transporters

RESULTS

Cloning of New Mammalian GABA Transporter Sequences:

We screened a rat brain cDNA library at low stringency with probes encoding the rat neuronal GABA transporter (GAT-1; (31)) in order to identify additional inhibitory amino acid transporter genes. Two clones were identified which hybridized at low but not at high stringency with the GABA transporter probes. DNA sequence analysis revealed that the clones encoded putative transporters which were

structurally related to GAT-1. The first clone, rB14b, contained a 2.0 kb sequence with an open reading frame of 1806 base pairs which could encode a protein of 602 amino acids (FIGS. 1A–1D). The second clone, rB8b (the truncated clone), contained a 1.9 kb sequence with an open reading frame of 1725 bp which could encode a protein of 575 amino acids. The nucleotide and amino acid sequences of rB8b (the truncated clone) are shown in FIG. 1B of U.S. Ser. No. 07/847,742, filed Mar. 4, 1992, now abandoned. Overlapping 45 mer oligonucleotides representing the 5' end of the rat GAT-3 clone were used to identify three rat genomic clones (rt12a, rt24a, and rt34a). A ~3.0 kb Pst I fragment of phage DNA isolated from rt24a was ligated into a plasmid vector (pUC18). Sequence analysis of the genomic fragment showed that there is a Met upstream in the genomic sequence and there is not a Met in the location where the rB8b (the truncated clone) has an ATG. Comparison of this genomic sequence with the sequence of another rat GAT-3 cDNA clone (rB13a) revealed that rB13a is only missing 2 bp at the 5' end. The missing sequence was filled in by ligation of synthetic oligonucleotides into a plasmid vector to form a construct designated K438 (clone K438-rB8b). Sequencing confirmed that the full-length rat GAT-3 DNA was present in K438-rB8b. K438-rB8b (the full-length clone) contained a 2.1 kb sequence which has an open reading frame of 1881 base pairs encoding a protein of 627 amino acids (FIGS. 2A–2D). The pharmacological characterization in U.S. Ser. No. 07/847,742 was carried out using rB8b (the truncated clone). Further experiments showed that K438-rB8b and rB8b exhibit similar pharmacology and as a result, the clones were used interchangeably. In the experiments of this invention designated for rat GAT-3, the data may have been generated using K438-rB8b or the data may have been generated using rB8b. When multiple individual experiments were pooled, the individual experiments could have been carried out with rB8b or K438-rB8b. rB14b and k438-rB8b exhibited 59% nucleotide identity throughout the coding region with the neuronal rat GABA transporter (GAT-1) and 70% nucleotide identity with each other. Comparison to sequences in Genbank and EMBL data bases demonstrated that both nucleotide sequences were novel and that the most homologous sequence was the rat GABA transporter GAT-1 (31). Subsequent comparisons which included recently cloned transporters revealed that the most closely related sequence is the canine betaine transporter (110) which exhibits 69% nucleotide identity with both rB14b and k438-rB8b. The taurine transporter (96) and the glycine transporter (98) are also significantly related, exhibiting ~64% and ~56% nucleotide identity, respectively, to both rB14b and k438-rB8b.

The amino acid sequence deduced from the nucleotide sequence of rB14b is shown in FIGS. 4A–4B modeled after the proposed membrane topology of GAT-1 (31). Residues identical to those in k438-rB8b are shaded and represent 67% amino acid identity between the two clones. The translation products of both rB14b and k438-rB8b are predicted to have relative molecular masses of ≈68,000 Daltons. Hydropathy analyses indicate the presence of 12 hydrophobic domains in both proteins which may represent membrane spanning segments. For each transporter, several potential sites for Asn-linked glycosylation are found in the extracellular loop between the third and fourth transmembrane domains. Comparison and alignment of the deduced amino acid sequences of rB14b (GAT-2) and k438-rB8b (GAT-3) with the neuronal GABA transporter (GAT-1) (FIGS. 6A–6D) revealed 52.5% and 52% amino acid identities, respectively. The betaine transporter (FIGS. 6A–6D), which can also transport GABA (110) exhibited a significantly higher degree of homology—68% and 65%

amino acid identities to rB14b and K438-rB8b, respectively. Similarly, the transporter for taurine (96), an inhibitory amino acid, is 61% homologous to both. In contrast, comparison of the new transporters with the rat glycine transporter (FIGS. 6A–6D and Ref.(98)) or the human norepinephrine transporter (79) showed a lower degree of amino acid identity (43–45%), similar to that between the neuronal GABA and norepinephrine transporters (46%). These data suggested that the new sequences might encode additional amino acid transporters expressed in the brain. To explore this possibility, the sequences were each placed in a mammalian expression vector, transfected into COS cells, and screened for transport of a variety of radiolabeled neurotransmitters and amino acids. These studies revealed (see below) that rB14b and K438-rB8b encode novel GABA transporters with pharmacological properties distinct from the neuronal GABA transporter.

Pharmacological Characterization of Mammalian GABA Transporters:

COS cells transiently transfected with rB14b or, rB8b or K438-rB8b, (COS/rB14b and COS/rB8b or COS/K438-rB8b, respectively) accumulated more [³H]GABA than non-transfected control cells; representative experiments are shown in FIGS. 7A and 7B. During a 10 minute incubation (37° C.) with a low concentration of [³H]GABA, specific uptake was increased 52±11-fold (mean±SEM, n=6) and 64±12-fold (n=5) over control for rB14b and rB8b and/or K438-rB8b, respectively. In contrast the uptake of [³H] glutamate, [³H]glycine, [³H]5-HT, [³H]dopamine, and [³H] taurine was unaltered. Specific uptake represented greater than 95% of total uptake in transfected cells. Uptake of [³H]GABA was not observed following mock transfection or transfection with an irrelevant insert, indicating that the enhanced uptake was not the result of non-specific perturbation of the membrane. The transport of [³H]GABA by both COS/rB14b and COS/rB8b and/or COS/K438-rB8b was decreased >95% when Na⁺ was replaced by Li⁺ (Table 1); similar results were obtained with COS cells expressing GAT-1 (COS/GAT-1), which we re-cloned (see Materials and Methods). When Cl⁻ was replaced by acetate, [³H] GABA transport by COS/GAT-1 was nearly completely eliminated (Table 1), consistent with previous results obtained with this transporter (31,39). In contrast, transport by COS/rB14b and COS/rB8b and/or COS/K438-rB8b was decreased to 43 and 20% of control, respectively (Table 1). The difference in sensitivity to removal of chloride exhibited by the three transporters was statistically significant (GAT-1 vs. COS/rB14b, p<0.001; GAT-1 vs. rB8b and/or K438-rB8b, p<0.05; cB14b vs. rB8b and/or K438-rB8b, p<0.05).

To determine the affinity of GABA for the cloned transporters, COS/rB14b and COS/rB8b and/or COS/K438-rB8b were incubated with various concentrations of [³H] GABA and the specific accumulation of radioactivity was determined. Accumulation of [³H]GABA was dose-dependent and reached saturation at higher concentrations (FIGS. 8A and 8B). Non-linear regression analysis of the data yielded the following values: K_M=8±3 μM and 12±6 μM, and V_{MAX}=2.5±1.2 and 3.0±0.9 nmoles/mg protein for COS/rB14b and COS/rB8b and/or COS/K438-rB8b, respectively (mean±SEM, n=4 experiments). Taken together, these data indicate that both rB14b and rB8b and/or K438-rB8b encode saturable, high-affinity, sodium- and chloride-dependent GABA transporters. Accordingly, we propose the terms GAT-2 and GAT-3 for the transporters encoded by rB14b and rB8b and/or K438-rB8b, respectively, according to the nomenclature proposed by Guastella et al. (31).

To determine the pharmacological properties of the cloned GABA transporters, we examined the ability of

various drugs to inhibit the accumulation of [³H]GABA by GAT-2 and GAT-3; for comparison, we also examined the pharmacology of GAT-1. As shown in Table 2, the pharmacological properties of GAT-2 and GAT-3 are similar to one another, but differ considerably from GAT-1. For example, β-alanine, a ligand reported to be selective for glial GABA transport (47), is more potent at the new cloned transporters than at GAT-1. In contrast, ACHC, guvacine, nipecotic acid, and hydroxynipecotic acid are more potent at GAT-1 than at GAT-2 and GAT-3. Interestingly, the two newly cloned transporters can be distinguished by L-DABA which displays high affinity for GAT-2 as well as GAT-1, but is less potent at GAT-3.

To further characterize the pharmacological properties of GAT-2 and GAT-3, we examined the ability of (R)-Tiagabine and CI-966 to inhibit the uptake of [³H]GABA; for comparison, we also examined these compounds at GAT-1. These compounds are lipophilic derivatives of nipecotic acid and guvacine, respectively. As shown in Table 2, (R)-Tiagabine at a concentration of 10 μM completely inhibits uptake at GAT-1 but has no effect at GAT-2 and GAT-3. Tiagabine is reported to have high potency at both neuronal and glial GABA transporters (13), and has demonstrated efficacy as an anticonvulsant in early clinical trials (16). The finding that Tiagabine has very low affinity for GAT-2 and GAT-3 underscores the potential of these transporters as unique drug targets. Similar to Tiagabine, the GABA uptake blocker CI-966 (104) displays far greater potency at GAT-1 than at GAT-2 and GAT-3 (Table 2). CI-966 was developed as an anticonvulsant but was withdrawn due to severe side effects observed in Phase 1 clinical trials (93).

TABLE 1

Condition ^a	Ion Dependence of [³ H]GABA Uptake		
	Uptake ^a		
	GAT-1	GAT-2	GAT-3
Na ⁺ -free	0.5 ± 0.3 (3)	0.1 ± 0.06 (3)	0.3 ± 0.03 (3)
Cl ⁻ -free	5 ± 2 (3)	43.2 ± 4.0 (5)	20.2 ± 5.8 (5)

^aCOS-7 cells transfected with rB46a, rB14b or K438-rB8b, or rB8b were incubated for 10 minutes (37° C.) with 50 nM [³H]GABA in either HBS, or in HBS in which Li⁺ was substituted for Na⁺ (Na⁺-free), or in which acetate was substituted for Cl⁻ (Cl⁻-free). Non-specific uptake was determined with 1 mM GABA. Data represent specific uptake, expressed as percent of uptake in HBS (mean ± SEM; values in parentheses indicate number of experiments)

TABLE 2

Inhibitor ^a	concentration	Pharmacological Specificity of [³ H]GABA Uptake		
		% Inhibition ^a		
		GAT-1	GAT-2	GAT-3
ACHC ^b	100 μM	49 ± 10(3)	3 ± 3(3)	0 ± 0(3)
β-alanine	100 μM	11 ± 1(8)	86 ± 1(8)	70 ± 1(7)
betaine	500 μM	0(2)	9(2)	1(2)
L-DABA	100 μM	49 ± 8(7)	43 ± 8(7)	4 ± 1(5)
guvacine	10 μM	41 ± 3(4)	13 ± 1(3)	8 ± 5(3)
OH-nipecotic	10 μM	34 ± 5(3)	9 ± 7(3)	5 ± 2(3)
nipecotic	10 μM	51 ± 5(3)	5 ± 5(3)	12 ± 6(3)
THPO	100 μM	10(2)	9(2)	4(2)

5

10

15

20

25

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40

45

50

55

60

65

TABLE 2-continued

Inhibitor ^a	concentration	Pharmacological Specificity of [³ H]GABA Uptake % Inhibition ^a		
		GAT-1	GAT-2	GAT-3
(R)-Tiagabine	100 μ M	100 $\pm$ 1(3)	0 $\pm$ 1(3)	0 $\pm$ 1(3)
CI-966	100 μ M	91 $\pm$ 2(3)	9 $\pm$ 6(3)	10 $\pm$ 6(3)

^aCOS-7 cells transfected with rB46a, rB14b or K438-rB8b, or rB8b were incubated for 10 minutes (37° C.) with 50 nM [³H]GABA and the indicated compounds. Non-specific uptake was determined with 1 mM GABA. Data show percent displacement of specific [³H]GABA uptake, mean $\pm$ SEM (values in parentheses indicate number of experiments).
^bL-DABA = L-(2,4)diaminobutyric acid
THPO = 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol
ACHC = cis-3-aminocyclohexanecarboxylic acid
CI-966 = [1-[2-[bis 4-(trifluoromethyl)phenyl]methoxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid
Tiagabine = (R)-N-[4,4-bis(3-methyl-2-thienyl)but-3-en-1-yl]nipecotic acid

LIPOPHILIC GABA TRANSPORT INHIBITORS
SK&F 89976-A, TIAGABINE, CI-966, AND
NNC-711 ARE SELECTIVE FOR THE CLONED
GABA TRANSPORTER GAT-1

To gain a better understanding of the contribution of the cloned GABA transporters to neuronal and glial transport systems, we have undertaken a combined pharmacological and molecular biological study using cell cultures derived from rat brain. We first defined the pharmacological profile of the four cloned GABA transporters (i.e., GAT-1, GAT-2, GAT-3, and BGT-1), and a related member of this family, the taurine transporter (TAUT; Smith et al., 1992). Next, we examined GABA transport activity in neuronal cultures, and in both Type 1 and O-2A/Type 2 astrocytic cell cultures, and compared their profiles to those of the cloned transporters. Last, we examined these cultures for the presence and relative abundance of mRNA for each of the four GABA transporters and TAUT.

In the present study we used transfected cell lines expressing rat and human GAT-1, rat GAT-2, rat and human GAT-3, and human BGT-1. FIG. 16 shows a dendrogram describing the amino acid relationships between these transporters. As described previously (Borden et al., 1992), GAT-2 and GAT-3 are more closely related to each other than to GAT-1. Human BGT-1 is most related to GAT-2, and least to GAT-1. As expected, species homologues are highly related to one another.

FIG. 17 shows the structures of the lipophilic compounds examined in this study, as well as the parent compounds ($\pm$)-nipecotic acid and guvacine. SK&F 89976-A is a nipecotic derivative with a 4,4-diphenyl-3-butenyl moiety

attached to the ring nitrogen (Yunger et al., 1984). Tiagabine is similar to SK&E 89976-A but has a bis-3-methylthienyl substituent instead of the two phenyl groups (Nielsen et al., 1991) CI-966 is a guvacine derivative and differs from the previous two compounds in having a para-substituted trifluoromethyl biphenyl ether moiety attached to the ring nitrogen via an ethyl bridge (Bjorge et al., 1990). NNC-711 is also a guvacine derivative with a biphenylmethylenoxime unit attached to the ring nitrogen, also via an ethyl bridge.

Table 3 shows the potency of GABA and the hydrophilic inhibitors ($\pm$)-nipecotic acid, guvacine, and β -alanine at each of the four cloned GABA transporters. GABA displays similar high-affinity ($IC_{50}\approx 5 \mu$ M) at human GAT 1, rat GAT-2, and human GAT-3, but is approximately 7-fold less potent at human BGT-1. The lower affinity of GABA at rat GAT-1 and rat GAT-3 is due, at least in part, to the use of transient transfection for these clones and perhaps to species differences in transporter structure that may alter drug affinities. Table 3 also shows that ($\pm$)-nipecotic acid and guvacine display modest selectivity for GAT-1 ($IC_{50}s=14-39 \mu$ M) as compared to GAT-2 and GAT-3 ($IC_{50}s=102-378 \mu$ M), whereas both compounds have low affinity at hBGT-1 ($IC_{50}>1$ ml). As described previously (Borden et al., 1992), β -alanine has greater potency at GAT-2 and GAT-3 ($IC_{50}s=19-110 \mu$ M) than at GAT-1, regardless of species; β -alanine also displays low potency ($IC_{50}>1$ mM) at hBGT-1.

Table 4 shows the summarized data for the potency of the lipophilic compounds at the cloned transporters, and a representative experiment for Tiagabine is shown in FIG. 18. The IC_{50} values of the lipophilic compounds at GAT-1 range between 26 and 260 nM at GAT-1 (Table 4); these compounds are thus 50- to 200-fold more potent at this transporter than are ($\pm$)-nipecotic acid and guvacine. The compound with the highest affinity at human GAT-1 is NNC-711, followed by Tiagabine, SK&F 89976-A, and CI-966. A similar order of potency is observed at rat GAT-1 although the absolute affinities are somewhat lower.

In contrast to GAT-1, the lipophilic compounds all display low affinity at GAT-2, GAT-3, and BGT-1. As shown in Table 4, the IC_{50} values at these transporters range from about 300 μ M to greater than 1 mM, regardless of species (rat or human) or type of transfectant (stable or transient). Thus, all four lipophilic compound, display specificity for GAT-1 of nearly three orders of magnitude when compared to the other cloned GABA transporters. These findings confirm and extend the report by Clark et al. (1992) that NNC-711 has higher affinity for GAT-1 than GAT-3.

TABLE 3

Affinity of hydrophilic transport inhibitors at cloned GABA transporters. The compounds shown were examined for their ability to inhibit uptake of [³ H]GABA by each of the cloned GABA transporters, as described in Methods. Data show the IC_{50} for inhibition of [³ H]GABA uptake, in μ M, and represent means $\pm$ SEM; the values in parentheses indicate the number of experiments.						
Clone/ compound	IC_{50}, μ M					
	human GAT-1	rat GAT-1	rat GAT-2	human GAT-3	rat GAT-3	human BGT-1
GABA	5 $\pm$ 1 (3)	30 $\pm$ 8 (3)	5 $\pm$ 2 (3)	7 $\pm$ 1 (4)	33 $\pm$ 10 (3)	36 $\pm$ 3 (4)
($\pm$)-nipecotic	8 $\pm$ 0.4	24 $\pm$ 6	38 $\pm$ 4	106 $\pm$ 13	159 $\pm$ 30	2370 $\pm$ 617

TABLE 3-continued

Affinity of hydrophilic transport inhibitors at cloned GABA transporters.
The compounds shown were examined for their ability to inhibit uptake of [³H]GABA by each of the cloned GABA transporters, as described in Methods. Data show the IC₅₀ for inhibition of [³H]GABA uptake, in μ M, and represent means $\pm$ SEM; the values in parentheses indicate the number of experiments.

Clone/ compound	human GAT-1	rat GAT-1	rat GAT-2	human GAT-3	rat GAT-3	human BGT-1
acid	(3)	(3)	(4)	(3)	(3)	(4)
guvacine	14 $\pm$ 3	39 $\pm$ 6	58 $\pm$ 5	119 $\pm$ 24	378 $\pm$ 175	1870 $\pm$ 387
β -alanine	(3)	(3)	(4)	(3)	(3)	(3)
	5690 $\pm$ 1890	2920 $\pm$ 197	19 $\pm$ 7	58 $\pm$ 3	110 $\pm$ 40	1320 $\pm$ 224
	(3)	(3)	(3)	(3)	(3)	(3)

TABLE 4

Affinity of lipophilic transport inhibitors at cloned GABA transporters.
The compounds shown were examined for their ability to inhibit uptake of [³H]GABA by each of the cloned GABA transporters, as described in Methods. Data show the IC₅₀ for inhibition of [³H]GABA uptake, in μ M, and represent means $\pm$ SEM; the values in parentheses indicate the number of experiments.

Clone/ compound	human GAT-1	rat GAT-1	rat GAT-2	human GAT-3	rat GAT-3	human BGT-1
CI-966	0.26 $\pm$ 0.05	1.2 $\pm$ 0.2	297 $\pm$ 34	333 $\pm$ 76	1140 $\pm$ 337	300 $\pm$ 10
	(4)	(5)	(3)	(3)	(3)	(3)
Tiagabine	0.07 $\pm$ 0.007	0.64 $\pm$ 0.15	1410 $\pm$ 250	917 $\pm$ 193	2040 $\pm$ 107	1670 $\pm$ 722
	(7)	(5)	(3)	(3)	(3)	(3)
SK&F 89976-A	0.13 $\pm$ 0.01	0.64 $\pm$ 0.19	550 $\pm$ 225	944 $\pm$ 259	4390 $\pm$ 1420	7210 $\pm$ 3630
	(3)	(5)	(4)	(3)	(3)	(4)
NNC-711	0.04 $\pm$ 0.01	0.38 $\pm$ 73	171 $\pm$ 53	1700 $\pm$ 252	349 $\pm$ 151	622 $\pm$ 265
	(4)	(4)	(5)	(3)	(3)	(4)

Tissue Localization Studies of Mammalian GABA Transporters:

To define the tissue distribution patterns of the novel GABA transporters, polymerase chain reaction (PCR) was used to detect each sequence in cDNA from seven different rat tissues. For comparison, the distribution of GAT-1 was also studied. Radiolabeled probes were used to detect individual PCR products by hybridization; each of the probes was highly specific for the transporter under study (data not shown). As shown in FIG. 9B, GAT-1 was detectable in brain and retina but not liver, kidney, heart, spleen, or pancreas after 30 cycles of PCR. GAT-2 was present not only in brain and retina, but also in liver, kidney, and heart. Levels of GAT-2 mRNA were also detectable in spleen with over-exposure of the autoradiogram (data not shown). Similar to GAT-1, the distribution of GAT-3 was limited to brain and retina. Cyclophilin was amplified to a similar extent from all the tissues (data not shown), indicating that adequate cDNA was present in each sample. Samples of poly A+ RNA not treated with reverse transcriptase and subjected to identical PCR conditions showed no hybridization with the transporter probes (not shown), indicating that the signals obtained with cDNA could not be accounted for by genomic DNA contamination. Thus, among the tissues examined, the distribution of GAT-3 is limited to the CNS, while GAT-2 has a wide peripheral distribution as well. These results are supported by Northern blot analyses of total RNA isolated from rat brain and liver; a single $\approx$ 2.4 kb transcript hybridizing with GAT-2 is present in both liver and brain, while a $\approx$ 4.7 kb transcript hybridizing with GAT-3 is detectable only in brain (FIG. 9A).

Cellular Localization of GABA Transporter RNAs:

Prior to the recent cloning of GABA transporters (9,31), pharmacological evidence suggested that multiple transporters contributed to the high-affinity GABA uptake observed in rat brain (40). Both neuronal and glial elements transport GABA, and preparations enriched in each cell type display differential sensitivities to inhibitors of GABA transport (12, 75, 91), suggesting the presence of distinct neuronal and glial GABA transporters. The ability to design neuronal- or glial-selective GABA uptake inhibitors would be a major advantage in the design of effective therapeutic agents. The GABA transporter cloned from rat brain, designated GAT-1 (31), displays a pharmacological profile consistent with a “neuronal”-type carrier. Our cloning of two additional GABA transporters from rat brain, GAT-2 and GAT-3 (previously termed Ggaba1 and Ggaba2, respectively), confirms the principle of heterogeneity in high-affinity GABA transporters. Further, the sensitivity of GAT-2 and GAT-3 to inhibition by β -alanine distinguishes them from GAT-1, and raises the possibility that one or both represent “glial”-type transporters. The availability of three cloned high-affinity GABA transporters now provides the opportunity to begin to examine the relationship between the pharmacologically defined neuronal and glial subtypes, and the transporters encoded by the cloned genes.

The presence of mRNAs representing each of the three GABA transporters was investigated in primary cultures of embryonic rat brain neurons, astrocytes, and meningeal fibroblasts. Polymerase chain reaction (PCR) was used to amplify each sequence for detection with specific probes. As

shown in Table 5, the messenger RNAs encoding each GABA transporter had a unique pattern of distribution. GAT-1 mRNA was present in all three culture types, whereas GAT-3 mRNA was restricted to neuronal cultures. GAT-2 mRNA was present in both astrocyte and fibroblast cultures, but not in neuronal cultures. Thus, GAT-2 and GAT-3, which exhibit extremely similar pharmacological profiles, display non-overlapping cellular distribution patterns. GAT-1, which displays a “neuronal”-type pharmacology, is apparently not restricted to a neuronal distribution.

TABLE 5

Cellular Localization of GABA Transporters by PCR.			
	Neuronal Cultures	Astrocyte Cultures	Fibroblast Cultures
GAT-1	+	+	+
GAT-2	-	+	+
GAT-3	+	-	-

Total RNA isolated from cultured embryonic rat neurons, astrocytes, or fibroblasts was converted to cDNA and subjected to PCR for detection of mRNAs encoding GAT-1, GAT-2, and GAT-3 as described in Experimental Procedures. Amplified products were separated on agarose gels, blotted to nylon membranes, and hybridized with radiolabeled oligonucleotides specific for each transporter cDNA. The blot was exposed to film and the autoradiogram developed after several hours. A (+) sign signifies that a positive signal was detected on the autoradiogram; a (-) signifies that no signal was detectable. The same results were observed in two independent experiments.

It is important to note that primary cultures, while enriched for a specific population of cells, may contain a small proportion of additional cell types. The sensitivity of PCR is sufficient to amplify a sequence contributed by a small number of cells; therefore, an unequivocal assignment of neuronal vs. glial localization would require combined in situ hybridization/immunocytochemistry. However, the presence of GAT-3 mRNA only in neuronal cultures suggests that detection of GAT-1 mRNA in astrocyte cultures is not due to the presence of contaminating neurons, and that GAT-1 is probably present in astrocytes in addition to neurons. The presence of GAT-1 and GAT-2 in fibroblast as well as astrocyte cultures may be explained by our recent finding that meningeal fibroblast cultures contain a large proportion of astrocytes as defined by staining with antibodies to glial fibrillary acidic protein (GFAP) (data not shown); thus, GAT-1 and GAT-2 signals in meningeal fibroblasts probably result from contaminating astrocytes.

These studies suggest that multiple high-affinity GABA transporter subtypes are present in different functional compartments, with at least two subtypes present in neurons (GAT-1 and GAT-3) and in Glia (GAT-1 and GAT-2). Further, they indicate that pharmacologic agents selective for each subtype may have different therapeutic applications.

Localization of GAT-1 and GAT-3 mRNA by in situ Hybridization:

In situ hybridization of GAT-1 and GAT-3 was carried out using antisense probes to the 3' untranslated region and the 3,4 extracellular loop of each clone. Hybridization of sense probes (control) to the same regions were also studied.

GAT-1 mRNA was observed in all rat brain areas examined (Table 6). In the telencephalon, the highest levels were observed in the glomerular layer of the olfactory bulb, the orbital cortex, the lateral septal nucleus, the ventral

pallidum, the globus pallidus, amygdaloid area, and layer 4 of the cerebral cortex. Moderate levels were observed in the islands of Calleja, the internal and external plexiform layers, and the piriform, retrosplenial, and cingulate cortices, as well as in all regions of the hippocampal formation.

In the diencephalon, the highest levels were found in the paraventricular and reticular thalamic nuclei, and in the dorsal lateral geniculate. Lower levels were seen in the reuniens and rhomboid thalamic nuclei. In the hypothalamus, moderate levels were seen in the suprachiasmatic and paraventricular nuclei, and in the medial pre-optic area. Lower levels were seen in the supraoptic and anterior hypothalamic nuclei.

TABLE 6

In situ localization of GAT-1 in the Rat CNS		
Area ¹ loop	Labeling ²	
	Probe 191 AS 3'UT	Probe 179 AS 3,4
BREGMA 6.20 mm		
mitral cells	-	-
glomerular layer	++	++
ext. plexiform layer	+½	+
ant. olf nerve	+/-	+/-
BREGMA 5.20 mm		
ext. plexiform layer	+	+
int. plexiform layer	+	+
ant. comm. intrabulb	+/-	+/-
AOM, D, V	+	+
orbital cortex m, v, l	+½	+½
frontal. cortex	+	+½
BREGMA 1.60 mm		
tenia tecta	+	+
lat. septal nucleus	+/-	+/-
lat. septal interm.	++	½+
ICjM	+½	+½
caudate-putamen	+/-	-
AcbSh	+	½+
AcbC	½+	-
vent. pallidum	+++	+++
olf. tubercle	-	-
ICj	+	+
piriform ctx.	+	+
cingulate ctx	+	+
indusium griseum	++	+½
BREGMA -1.40 mm		
retrosplen. ctx	+	½+
cortex I	+	+
cortex IV	++	++
cortex V	+	+
reticular thal. nu.	+½	+½
globus pallidus	+++	++½
caudate-putamen	+	+
ant. dor thal. nu.	-	-
paravent. thal. nu	+½	+½
supraoptic nu.	½+	½+
suprachiasmatic nu.	+	+
med. preoptic area	+½	+½
perivent. hypoth. nu.	+	+
anter. hypoth. nu.	+	+
paravent. hypoth. nu.	+½	+½
nu. horizontal. limb		
diag. band	+	+
ant. amygd. area	++½	++½
BREGMA -1.80 mm		
reuniens thal. nu.	½+	½+
rhomboid thal. nu.	½+	½+
retrochiasmatic area	+	+

TABLE 6-continued

In situ localization of GAT-1 in the Rat CNS		
Area ¹ loop	Labeling ²	
	Probe 191 AS 3'UT	Probe 179 AS 3,4
<u>BREGMA -4.52</u>		
choroid plexus	-	-
PMCo	+	+
AHiA	+	+
Basolateral Amygdaloid nu.	++	++
dorsal endopiriform nu.	+	+
hippocampus (all levels)	+	+
polymorphic dentate gyrus	++	++
olivary pretectal nu.	++	++
dorsal lateral genicul. nu.	++	++
<u>BREGMA -5.30 mm</u>		
<u>substantia nigra</u>		
pars reticulata	++	++½
pars compacta	++	++
red nucleus parvocellular	-	-
retrosplenial cortex	+	+
occipital cortex	+	+
nucleus Darkschewitsch	+½	+
nucleus posterior commis., magnocellular	+	+½
<u>BREGMA -7.64 mm</u>		
superior colliculus	+	+
central grey	-	-
dorsal grey	+/-	+/-
median Raphe	+½	+½
pontine nuclei	-	-
Purkinje cells	+/-	+/-

¹abbreviations as in Paxinos, G. and Watson, C. (1986) The Rat Brain in Stereotactic Coordinates, second edition. Academic Press.
²Antisense probes 191 and 179 were to 3' untranslated region and to the 3,4 extracellular loop, respectively. Control data using sense probes to the same regions showed no labeling.
Labeling scale: -, no labeling; ½+, very weak; +, weak; ++, moderate; +++, heavy. Note that the scale is based on maximal labeling obtained with GAT-1 probes and should not be compared to results for GAT-3.

In the midbrain, high levels were seen in the substantia nigra (pars compacta and pars reticulata), median raphe, and the olivary pretectal nucleus. Lower levels were observed in the superior colliculus.

No label was seen in the pontine nuclei, nor in the cerebellar Purkinje cells.

GAT-3 mRNA was observed throughout the neuraxis (Table 7). Within the telencephalon, the highest levels were detected in the medial septal nucleus, the nucleus of the diagonal band, and the ventral pallidum. Lower levels were found in the amygdala and the shell of the nucleus accumbens. Low levels were observed in the hippocampus. No labeling above background was observed in the neocortex.

In the thalamus, many nuclear groups were labeled. The areas with the highest labeling were the xiphoid, paraventricular, and rhomboid nuclei, and the zona incerta. Lower levels were observed in the following nuclei: reuniens, reticular, medial and lateral ventral posterior and the medial geniculate. In the hypothalamus, moderate labeling was found in the lateral and ventromedial regions. Lower levels were observed in the arcuate nucleus and median eminence.

In the midbrain, the highest levels were observed in the dorsail tegmentum.

In the metencephalon, the highest levels were found in the medial vestibular and deep cerebellar nuclei, and lower levels in the lateral superior olivary nucleus.

No label was observed in the cerebellar cortex.

A comparison of the localization of GAT-1 and GAT-3 mRNAs indicates that both are widely distributed in the brain, and while GAT-1 is more abundant on a per cell basis, the two tend to have overlapping distributions. Notable exceptions are cortex and hippocampus which contain large numbers of neurons containing GAT-1 mRNA but few cells with GAT-3 mRNA. On the other hand, GAT-3 mRNA levels appear to be higher than GAT-1 in the superficial layers of the superior colliculus and in the deep cerebellar nuclei.

TABLE 7

In situ Localization of GAT-3 in the Rat CNS	
Area ¹	Labeling ²
<u>telencephalon:</u>	
cortex	-
piriform ctx	½+
<u>nu. accumbens</u>	
core	-
shell	+
olf. tubercle	½+
med. septal nu.	++
nu. horiz. limb diag. band	++
ventral pallidum	++
ant. cortical amygdaloid nu.	+
medial amygdaloid nu.	+½
<u>Diencephalon:</u>	
paraventricular thalamic nu.	++½
reticular thalamic nu.	+½
VPL	+
VPM	½+
zona incerta	++½
rhomboid thalamic nu.	++½
reuniens thalamic nu.	++
xiphoid thalamic nu.	+++
medial geniculate nu.	+
arcuate hypoth. nu.	½+
ventromedial hypoth. nu.	+
lateral hypoth. nu.	+½
median eminence	½+
hippocampus	½+
<u>Mesencephalon:</u>	
superior colliculus	++½
central gray, dorsal	++
central gray	++
substantia nigra	not examined
interpeduncular nu. caudal	+
dorsal raphe	+
cuneiform nu.	+
lateral dorsal tegmen. nu.	+++
dorsal tegmental nu., pericentral	+++
<u>Metencephalon:</u>	
medial vestibular nu.	+++
lateral superior olive	++
inferior olive	not examined
cerebral cortex	-
deep cerebellar nuclei	+++

¹abbreviations as in Paxinos, G. and Watson, C. (1986) The Rat Brain in Stereotaxic Coordinates, second edition. Academic Press.
²Data are pooled from antisense probes to the 3' untranslated region and to the 3,4 extracellular loop. Control data using sense probes to the same regions showed no labeling.
Labeling scale: -, no labeling; ½+, very weak; +, weak; ++, moderate; +++, heavy. Note that the scale is based on maximal labeling obtained with GAT-3 probes and should not be compared to results for GAT-1.

GABA TRANSPORT IN NEURONAL AND
GLIAL CELL CULTURES: CORRELATION OF
PHARMACOLOGY AND mRNA
LOCALIZATION

Pharmacological Characterization of Cloned GABA Trans-
porters:

In an attempt to identify the GABA transporters in neuronal and glial cell cultures, we adopted a two-pronged strategy: comparison of their pharmacological profiles with those of cloned transporters, coupled with mRNA localization. To avoid possible complications resulting from species differences, we used GAT-1, GAT-2, GAT-3 and TAUT cloned from rat brain, the same species from which the neuronal and glial cell cultures were prepared. As the rat BGT-1 transporter had not yet been cloned, we used PCR to isolate a fragment of it for use in Northern blot analysis of mRNA (see Methods). However, since the full-length clone of rat BGT-1 was not available, we chose to study the pharmacologic properties of a human betaine/GABA transporter (hBGT-1) which we recently cloned from a human brain cDNA library.

In our original description of GAT-2 and GAT-3 (Borden et al., 1992) and TAUT (Smith et al., 1992), we examined inhibitors at limited concentrations. We have now conducted an extensive analysis using 11 drugs which are known inhibitors of GABA and/or taurine transport. Full competition curves were obtained for each drug using 50 nM [^{3} H]GABA for the high-affinity GABA transporters (GAT-1, GAT-2, GAT-3, and BGT-1) and 50 nM [^{3} H]taurine for TAUT. The results of these studies are summarized in Table 8.

Under the conditions employed, GABA shows similar affinity at all four cloned GABA transporters (Table 8). GAT-1 is distinguished by high affinity for NNC-711 and ACHC, and low affinity for β -alanine. The rank order of potency is NNC-711>nipecotic acid>GABA>guvacine>($\pm$)-hydroxynipecotic acid>L-DABA $\approx$ ACHC>hypotaurine> β -alanine>GES>taurine. The pharmacologic profile of GAT-1 is quite dissimilar from that of the other cloned transporters as can be seen from Table 9, which shows the correlation of the pIC₅₀ (-log IC₅₀) values of the various cloned transporters with one another. The most selective GAT-1 compound (NNC-711, 1,000-fold) proved useful in distinguishing GAT-1-mediated GABA transport from that mediated by other GABA transporters in neuronal and glial cultures, and in brain aggregates (see below).

GAT-2 and GAT-3 are pharmacologically similar to one another ($r=0.97$) but distinct from the other cloned transporters (Table 9). In contrast to GAT-1, GAT-2 and GAT-3 have higher affinity for β -alanine than for ACHC and NNC-711. The order of potency for GAT-2 is GABA>hypotaurine> β -alanine>($\pm$)-nipecotic acid>guvacine>L-DABA>hydroxynipecotic acid>GES>NNC-711>taurine>ACHC, while that for GAT-3 is GABA>hypotaurine> β -alanine>($\pm$)-nipecotic acid>guvacine>hydroxynipecotic acid>L-DABA>GES>taurine>ACHC. Hypotaurine, a compound classically described as an inhibitor of taurine transporter, is as potent at GAT-2 and GAT-3 as at TAUT.

The pharmacologic profile of hBGT-1 is distinct from that of the other transporters; a modest correlation is observed with GAT-2 and GAT-3 ($r=0.53$ and 0.50 , respectively; Table

9). The order of potency is GABA>L-DABA>hypotaurine>ACHC> β -alanine>guvacine>($\pm$)-nipecotic acid>NNC-711>hydroxynipecotic acid>GES>taurine. Though none of the drugs tested show selectivity for hBGT-1, a distinguishing feature is the similar potency of β -alanine and ACHC.

We also examined the profile of TAUT using [^{3} H]taurine. The order of potency is hypotaurine=taurine>GES> β -alanine>NNC-711>GABA>guvacine>($\pm$)-nipecotic acid>hydroxynipecotic acid $\rightarrow$ ACHC>L-DABA. The overall profile correlates poorly with the other transporters (Table 9), though TAUT is similar to GAT-2 and GAT-3 in its preference for β -alanine over ACHC. Importantly, the high affinity for taurine distinguishes TAUT from the other transporters.

Preparation of Neuronal and Glial Cell Cultures:

To examine GABA transport in neurons and astrocytes, we prepared cell cultures highly enriched in each of these cell types (see Methods). Neuronal cultures were established from E17 embryonic brain and were treated with cytosine β -D-arabinofuranoside to inhibit the proliferation of non-neuronal cells (i.e., Gila and fibroblasts). After one week in vitro the cultures consisted of highly differentiated neurons displaying an intricate network of processes, which stained positively with an anti-neurofilament antibody (FIG. 19A).

Raff and colleagues have demonstrated that two classes of astrocytes, designated Type 1 and Type 2, can be identified in cell cultures derived from rat optic nerve (Martin et al., 1989; Raff, 1989). These cell types are morphologically and antigenically distinguishable, and are known to arise from distinct precursors (Lillien and Raff, 1990). Type 1 astrocytes are GFAP-positive, flat, cuboidal cells with a large, oval nucleus. In contrast, Type 2 cells are smaller, stellate cells with an eccentric nucleus and multiple processes (Vaysse and Goldman, 1990; Lillien and Raff, 1990). Type 2 cells are derived from an O-2A precursor cell which also gives rise to oligodendrocytes (Raff et al., 1983). Cultures highly enriched in each of these cell types were prepared as described in Methods, and are shown in FIGS. 19B (Type 1 astrocytes) and 19C (O-2A/Type 2 astrocytes).

Pharmacological Characterization of GABA Transporter Activity in Neuronal Cell Cultures:

Neuronal cultures avidly accumulated [^{3} H]GABA with specific uptake of 42 ± 4 pmoles per mg protein ($n=9$). Competition curves using unlabeled GABA were consistent with uptake occurring via a single high-affinity site, IC₅₀= 10 ± 2 μ M ($n=3$). The GAT-1-selective compound NNC-711 was a potent inhibitor (IC₅₀ $\approx$ 300 nM) of [^{3} H]GABA uptake but did not inhibit completely the specific uptake even at high concentrations (FIG. 20). When employed at 10 μ M, $31\pm 1\%$ ($n=6$) of the specific uptake persisted. This finding indicates that, under these conditions, about 30% of GABA uptake is mediated via a transporter(s) distinct from GAT-1. To characterize these sites we conducted competition curves in both the absence and presence of 10 μ M NNC-711, a concentration sufficient to inhibit transport via GAT-1 almost completely. We used the same 11 drugs used to define the properties of the cloned transporters (Table 8) to allow a direct comparison. As 70% of GABA uptake in neurons is mediated by GAT-1, uptake in the absence of NNC-711 ("NNC-711-sensitive") predominantly reflects transport via this site; in contrast, uptake in the presence of this blocker

("NNC-711-resistant") reflects transport via non-GAT-1 sites. The results of both sets of experiments are shown in Table 10. As expected, GABA transport in the absence of NNC-711 closely resembles the profile of GAT-1: Uptake is sensitive to NNC-711 (see above) and ACHC ($IC_{50} \approx 75 \mu M$), while β -alanine is a weak inhibitor ($IC_{50} \approx 4 mM$) (Table 10). The order of potency is NNC-711>GABA>guvacine>($\pm$)-nipecotic acid>hydroxynipecotic acid>ACHC>L-DABA>hypotaurine>GES> β -alanine>taurine, and the profile correlates well with GAT-1 ($r=0.99$) but poorly with the other transporters (Table 11). These data support the hypothesis that GAT-1 is the predominant GABA transporter in neuronal cultures.

We next examined the affinity of the same drugs in the presence of NNC-711. The affinity of GABA ($IC_{50}=10 \mu M$) is identical to that observed in the absence of NNC-711 (Table 10), consistent with our observation that GABA uptake in the absence of NNC-711 is well described by a single-site model (see above). Interestingly, the NNC-711-resistant GABA transport is more sensitive to β -alanine ($IC_{50} \approx 50 \mu M$) than to ACHC ($IC_{50} \approx 2 mM$), (Table 9), suggesting that the NNC-711-resistant component is mediated by GAT-2, and/or GAT-3, and/or TAUT. The order of potency is GABA>hypotaurine> β -alanine>($\pm$)-nipecotic acid>guvacine>hydroxynipecotic acid>GES>L-DABA>ACHC>taurine. As shown in Table 11, the highest degree of correlation is with GAT-2 ($r=0.87$) and GAT-3 ($r=0.904$). The correlation with hBGT-1 is modest ($r=0.69$), weaker with GAT-1, and poorest with TAUT. The contribution of these transporters was explored further by Northern blot analysis of neuronal mRNA (see below).

Pharmacological Characterization of GABA Transporter Activity in Glial Cell Cultures:

GABA transport was examined separately in Type 1 and O-2A/Type 2 astrocyte cultures. GABA transport in Type 1 cultures was low, averaging only 1.4 ± 0.3 pmoles/mg protein ($n=10$), which is about 3% of the uptake observed in neuronal cultures. The order of potency is guvacine>taurine>hypotaurine> β -alanine>GABA>GES>($\pm$)-nipecotic acid>ACHC>hydroxynipecotic acid>NNC-711>L-DABA (Table 10). The finding that GABA is a weak inhibitor ($IC_{50}=300 \mu M$) whereas taurine is a potent inhibitor ($IC_{50} \approx 50 \mu M$) suggests that GABA uptake in Type I astrocytes is mediated by TAUT. Consistent with this idea, the pharmacologic profile correlates most closely with TAUT, although the correlation is only modest ($r=0.52$); a poor correlation is observed with the other transporters (Table 11).

Unlike Type 1 astrocytes, O-2A/Type 2 astrocytes avidly accumulate [^3H]GABA (38 ± 4 pmoles/mg protein; $n=7$) with high affinity ($IC_{50} \approx 4 \mu M$, Table 10). NNC-711 is a potent inhibitor of GABA uptake ($IC_{50} \approx 70 nM$), but $26 \pm 4\%$ ($n=7$) of the specific uptake persisted in the presence of $10 \mu M$ NNC-711 (FIG. 20), similar to the situation in neurons. Accordingly, we examined the pharmacological profile of O-2A/Type 2 astrocytes both in the absence and presence of $10 \mu M$ NNC-711.

GABA transport in O-2A/Type 2 astrocytes, in the absence of NNC-711, displays high-affinity for NNC-711 ($IC_{50} \approx 7 nM$) and ACHC ($IC_{50} \approx 35 \mu M$), and low-affinity for D-alanine ($IC_{50} \approx 2 mM$) (Table 10). The order of potency is NNC-711>GABA>guvacine>($\pm$)-nipecotic

acid>hydroxynipecotic acid>ACHC>L-DABA>hypotaurine>GES> β -alanine>taurine, and there is an excellent correlation with GAT-1 ($r=0.99$, Table 11). Thus, in O-2A/Type 2 astrocytes, as in neurons, the majority of GABA transport is mediated via GAT-1.

GABA transport in O-2A/Type 2 astrocytes in the presence of $10 \mu M$ NNC-711 displays high-affinity for GABA ($IC_{50} \approx 2 \mu M$) and is more sensitive to β -alanine ($IC_{50} \approx 2 \mu M$) than to ACHC ($IC_{50} \approx 2 mM$) (Table 10). The order of potency is GABA> β -alanine>hypotaurine>($\pm$)-nipecotic acid>guvacine>L-DABA>hydroxynipecotic acid>taurine>GES>ACHC, and the data correlate best with GAT-2 ($r=0.96$) and GAT-3 ($r=0.94$) (Table 11). The correlation is modest with hBGT-1 ($r=0.69$), and poor with both GAT-1 and TAUT (<0.25 ; Table 11). To gain further information regarding the contribution of these transporters, we analyzed cultures for the presence of transporter mRNA (see below).

Pharmacological Characterization of GABA Transporter Activity in Brain Aggregates:

To examine GABA transport in freshly isolated rat brain, we employed a suspension assay utilizing dissociated brain aggregates. An advantage of this technique is that, unlike purification techniques which yield selective enrichment of various cell types, the brain aggregates include all the cellular elements in the brain in their original proportions.

Rat brain aggregates avidly transport [^3H]GABA (83 ± 12 pmoles/mg protein, $n=9$) with high affinity ($IC_{50} \approx 10 \mu M$; Table 12). NNC-711 is a potent inhibitor of GABA uptake ($IC_{50}=90 nM$), although $15 \pm 2\%$ ($n=3$) of the uptake persists in the presence of a high concentration ($10 \mu M$) of this drug (FIG. 21). Accordingly, we examined the pharmacological profile of brain aggregates both in the absence and presence of $10 \mu M$ NNC-711.

In the absence of NNC-711, ACHC ($IC_{50}=31 \mu M$) is more potent than β -alanine ($IC_{50} \approx 3 mM$; Table 12). The order of potency is NNC-711>($\pm$)-nipecotic acid>GABA=guvacine=hydroxynipecotic acid>L-DABA>ACHC>hypotaurine>GES> β -alanine>taurine, and there is an excellent correlation with GAT-1 ($r=0.99$) (Table 13). The NNC-711-resistant component of transport has high affinity for β -alanine ($IC_{50}=33 \mu M$) and low affinity for ACHC ($IC_{50} \approx 2.5 mM$). The order of potency is GABA>guvacine>hypotaurine> β -alanine>($\pm$)-nipecotic acid>hydroxynipecotic acid>L-DABA>NNC-711>GES>taurine>ACHC (Table 12), and the data correlate best with GAT-2 and GAT-3 ($r=0.96$ and 0.95 , respectively) (Table 13).

Localization of mRNA:

We next employed Northern blot analysis to examine the distribution of transporter mRNA in neuronal and glial cultures and in total brain. Probes for GAT-1, GAT-2, GAT-3 and TAUT were labeled to similar specific activities to allow a direct comparison, and Northern blots were reprobed for 1B15 (cyclophilin) for the purpose of normalization; however, as only a fragment of rBGT-1 was available the data obtained with this probe can be compared only semi-quantitatively to the other transporters (see Methods). Representative Northern blots are shown in FIGS. 23A–23J and the data are quantified in Table 14. As expected from the pharmacology, GAT-1 mRNA is abundant in neurons, whereas Northern blots failed to detect GAT-2 mRNA in

neuronal cultures. GAT-3 mRNA is also present in neuronal cultures but the levels are about one third those of neurons. mRNA for rBGT-1 is not detected by densitometry in neuronal cultures, though visual inspection of the autoradiograms sometimes reveals the presence of a faint band. TAUT mRNA is present at levels about 1.5-fold higher than GAT-1.

In Type 1 astrocyte cultures, mRNAs for GAT-1, GAT-2, and GAT-3 are not detectable by Northern blot analysis (FIGS. 23A–23J and Table 14). In contrast, mRNAs for rBGT-1 and TAUT are fairly abundant.

Levels of transporter mRNA expression in O-2A/Type 2 astrocytes are more variable than in the other cell types. GAT-1 mRNA was abundant in these cultures (FIGS. 23A–23J, Table 14), with levels approximately half those in neurons. GAT-2 mRNA is the most abundant GABA transporter with levels approximately 4-fold greater than GAT-1 mRNA. GAT-3 mRNA is present at levels about half those of GAT-1. mRNA for rBGT-1 and TAUT are also present in O-2A/Type 2 astrocytes, at levels similar to that of GAT-1. Thus, O-2A/Type 2 cultures contain all the known GABA transporters.

In summary, GAT-1 mRNA is present in neurons and O-2A/Type 2 astrocytes, as is GAT-3. GAT-2 mRNA is present in O-2A/Type 2 astrocytes, but not in neurons or Type 1 astrocytes. Type 1 astrocytes also lack GAT-1 and GAT-3. rBGT-1 mRNA is present in Type 1 and O-2A/Type 2 astrocyte cultures, but is rarely observed in neurons, and then at low levels. TAUT mRNA is present in all cell types examined, with the highest abundance in Type 1 astrocytes.

Using poly A⁺ RNA from total rat brain, all four high-affinity GABA transporters, and TAUT, were detected by Northern blots (not shown). GAT-1 and GAT-3 mRNAs were both quite abundant, while the levels of GAT-2 mRNA were about 20% of GAT-1 (Table 14). In contrast, BGT-1 and TAUT mRNA were relatively scarce, with levels ≈10% those of GAT-1 mRNA (Table 14).

Glial Nersus Neuronal Localization of the GABA Transporters:

Previous in situ hybridization studies had not demonstrated the presence of any of the GABA transporters in glial cells. There is now compelling evidence that the mRNA for at least one of the transporters, GAT-3, is localized to glial cells in select regions of the rat brain (see FIGS. 24A–24C). These regions include hypothalamus, superior colliculus, and some brainstem nuclei. This was accomplished by comparing the distribution of glial fibrillary acidic protein (GFAP), an immunological marker for some glial cells, to that obtained for GAT-3 mRNA. In the above areas, hybridization signal for GAT-3 mRNA is found over cellular profiles for which the size and staining characteristics are consistent with a glial localization. In addition, there is also hybridization signal over larger profiles which appear to be neuronal in nature. The functional significance of this is unclear at present. To some extent, this is consistent with the pharmacological profile obtained in cell cultures, and also with previous studies which have used immunologic probes for GABA transporter localization.

TABLE 8

Pharmacological profile of cloned GABA and taurine transporters. Data show the IC ₅₀ (in μM) for inhibition of [³ H]GABA uptake (GAT-1, GAT-2, GAT-3, hBGT-1) or [³ H]taurine uptake (TAUT) at the indicated cloned transporters. Values represent means ± SEM of at least three experiments.					
	IC ₅₀ , μM				
Clone/ drug	rGAT-1	rGAT-2	rGAT-3	hBGT-1	TAUT
GABA	30 ± 8	17 ± 6	33 ± 10	42 ± 12	720 ± 170
ACHC	132 ± 44	25,900 ± 12,600	55,100 ± 25,000	1,070 ± 508	9,230 ± 2,620
guvacine	39 ± 6	228 ± 38	378 ± 18	1,420 ± 201	1,030 ± 213
hydroxy-nipecotic	44 ± 8	567 ± 50	467 ± 53	3,840 ± 688	4,810 ± 653
(±)-nipecotic acid	24 ± 6	113 ± 33	159 ± 30	2,350 ± 253	2,030 ± 429
NNC-711	0.38 ± 0.07	729 ± 319	349 ± 151	3,570 ± 1,140	207 ± 59
hypotaurine	1,010 ± 135	52 ± 6	73 ± 7	536 ± 41	47 ± 5
β-alanine	2,920 ± 197	66 ± 9	110 ± 40	1,100 ± 66	176 ± 34
GES	4,840 ± 1,200	696 ± 110	915 ± 49	4,800 ± 910	129 ± 34
L-DABA	128 ± 12	300 ± 51	710 ± 237	528 ± 62	41,800 ± 29,100
taurine	32,300 ± 12,000	1,270 ± 40	2,860 ± 358	13,200 ± 1250	48 ± 11

TABLE 9

Correlation of GABA transport activity of cloned transporters.				
clone	rGAT-1	rGAT-2	rGAT-3	hBGT-1
rGAT-2	-0.07			
rGAT-3	0.22	0.97		
hBGT-1	0.27	0.53	0.50	
TAUT	-0.41	0.26	0.28	-0.26

The pIC₅₀ values for inhibition of [³H]GABA uptake (GAT-1, GAT-2, GAT-3, hBGT-1) or [³H]taurine uptake (TAUT) at each of the cloned transporters was correlated with the values at the other transporters (data from Table 1) Data represent r values.

TABLE 10

Pharmacological profile of GABA transport in rat neuronal and glial cell cultures. Data show the IC ₅₀ (in μM) for inhibition of [³ H]GABA uptake in neuronal and glial cultures, in the absence and presence of NNC-711. Values represent means ± SEM of at least three experiments.					
IC ₅₀ , μM					
assay condition/ drug	Neurons	Neurons with NNC-711 ¹	Type 1 astro- cytes	O-2A/ Type 2 astro- cytes	O-2A/ Type 2 astro- cytes with NNC-711 ¹
GABA	10 ± 2	10 ± 1	294 ± 100	4 ± 0.2	2 ± 0.4
ACHC	77 ± 8	1,950 ± 1,000	400 ± 200	35 ± 4	2,360 ± 500
guvacine	14 ± 2	136 ± 45	23 ± 10	8 ± 0.9	81 ± 30
hydroxy-nipecotic acid	22 ± 2	304 ± 100	1,610 ± 500	17 ± 3	197 ± 20
(±)-nipecotic acid	18 ± 5	119 ± 20	315 ± 100	12 ± 2	61 ± 20
NNC-711	0.28 ± 0.1	ND ²	2 ± 0.3	0.07 ± 0.01	ND
hypo-taurine	742 ± 200	25 ± 2	69 ± 30	620 ± 200	29 ± 15
β-alanine	4,060 ± 2,000	48 ± 10	100 ± 30	2,030 ± 300	24 ± 8
GES	2,110 ± 600	508 ± 60	310 ± 60	1,560 ± 300	603 ± 300
L-DABA	165 ± 20	861 ± 225	3,460 ± 2,000	74 ± 10	91 ± 30
taurine	8,300 ± 3,000	2,490 ± 900	47 ± 10	13,000 ± 6,000	328 ± 200

¹determined in the presence of 10 μM NNC-711

²not determined

TABLE 11

Correlation of GABA transport activity in neuronal and astrocyte cultures with cloned GABA and taurine transporters.					
clone/ assay condition	GAT-1	GAT-2	GAT-3	hBGT-1	TAUT
neurons	0.99	0.04	0.19	0.20	-0.35
neurons with NNC-711	0.36	0.87	0.90	0.69	0.24
Type 1 astrocytes	0.29	-0.12	-0.02	-0.16	0.52
O-2A/Type 2 astrocytes	0.99	0.07	0.22	0.30	-0.35
O-2A/Type 2 astrocytes with NNC-711	0.24	0.96	0.94	0.69	0.25

The pIC₅₀ values for inhibition of [³H]GABA transport in neuronal and astrocyte cultures (from Table 3) were correlated with the pIC₅₀ values at each of the cloned transporters (data from Table 1). Data represent r values.

TABLE 12

Pharmacological profile of GABA transport in rat brain aggregates.		
assay	IC ₅₀ , μM	
condition/	control	with NNC-711 ¹
GABA	10 ± 2	4 ± 0.8
ACHC	31 ± 4	2,590 ± 600
guvacine	10 ± 2	97 ± 30
hydroxy-nipecotic acid	10 ± 1	69 ± 10
(±)-nipecotic acid	7 ± 0.8	48 ± 10
NNC-711	0.09 ± 0.01	323 ± 70

TABLE 12-continued

Pharmacological profile of GABA transport in rat brain aggregates.		
assay	IC ₅₀ , μM	
condition/	control	with NNC-711 ¹
hypotaurine	692 ± 100	25 ± 10
β-alanine	2,930 ± 500	33 ± 8
GES	1,340 ± 100	410 ± 100
L-DABA	22 ± 3	144 ± 30
taurine	67,000 ± 20,000	730 ± 200

Data show the IC₅₀ (in μM) for inhibition of [³H]GABA uptake. Values represent means ± SEM of at least three experiments.

¹determined in the presence of 10 μM NNC-711

TABLE 13

Correlation of GABA transport activity in rat brain aggregates with cloned GABA and taurine transporters.					
clone/ assay condition	GAT-1	GAT-2	GAT-3	hBGT-1	TAUT
control	0.99	0.03	0.18	0.28	-0.50
10 μ M NNC-711	0.13	0.96	0.95	0.63	0.13

The pIC₅₀ values for inhibition of GABA transport in brain aggregates (data from Table 5) were correlated with the pIC₅₀ values at each of the cloned transporters (data from Table 1). Data represent r values.

TABLE 14

Quantitation of GABA transporter mRNA localization.				
cell culture/ clone	Neurons	Type 1 astrocytes	O-2A/Type 2 astrocytes	brain
GAT-1	1 ¹	0	0.4	1 ¹
GAT-2	0	0	1.7	0.2
GAT-3	0.3	0	0.2	1.2
BGT-1 ²	0	0.6	0.3	0.1
TAUT	1.6	2.2	0.6	0.1

Autoradiograms of Northern blots (such as those shown in FIGS. 23A-23J) were quantitated by densitometry, then corrected for the amount of mRNA as determined by hybridization to the constitutive mRNA encoding cyclophilin. Data are expressed as a fraction of GAT-1 mRNA present in neuronal cultures (for cell cultures), or total brain. Data for neurons, Type 1 astrocytes, and O-2/Type 2 astrocytes are means of 3 or 4 independent platings, assayed on separate gels.

¹by definition

²The specific activity of the rat BGT-1 probe was lower than that of the other probes (see Methods), thus the data underestimate the actual abundance

Discussion

The recent cloning of transporters for GABA (31) norepinephrine (79), dopamine (44,95), serotonin (8,33), glycine (98), and taurine (96) has helped to define the structural properties of this class of membrane proteins. In contrast with neurotransmitter receptors, however, it has not been determined for neurotransmitter transporters whether multiple subtypes exist and/or play a role in synaptic transmission. Our identification of two cDNA clones from rat brain encoding novel GABA transporters (designated GAT-2 and GAT-3) provides the first molecular evidence for heterogeneity within the neurotransmitter transporter gene family, and raises the possibility that multiple GABA transporters participate in the regulation of GABAergic neurotransmission.

Both proteins have 12 putative transmembrane domains and can be modeled with a similar topology to the neuronal GABA transporter (GAT-1; (31)), including a large glycosylated extracellular loop between TMs 3 and 4. Analysis of amino acid homologies of the various transporters reveals some unexpected relationships. For example, GAT-2 and GAT-3 exhibit greater amino acid sequence identity to each other (67%) than to GAT-1 (-53%), despite all three transporters displaying nearly identical affinities for GABA. Surprisingly, the sequence closest to GAT-2 and GAT-3 is the dog betaine transporter (110) which, in fact, is as homologous to GAT-2 and GAT-3 as they are to one another. Significantly, the cloned betaine transporter has also been reported to transport GABA (110), although the affinity of GABA at the betaine transporter is nearly 10-fold lower than at GAT-2 and GAT-3. conversely, the betaine transporter displays at least 10-fold higher affinity for betaine than do

GAT-2 and GAT-3 (see Table 2) Thus, transporters with as little as 53% amino acid homology can display high affinity for the same substrate (e.g. GAT-1 vs. GAT-2 and GAT-3), whereas transporters only slightly more divergent can demonstrate markedly different substrate specificities (e.g., GAT-1 vs. glycine, 45% homology;

Pharmacologically distinct GABA transporters have previously been identified in neuronal and glial cell cultures (24, 47 and 92). Thus, it was of interest to examine the sensitivity of GAT-2 and GAT-3 to a variety of inhibitors and to compare this to published values for endogenous transporters in primary cell cultures, as well as to GAT-1. It is noteworthy that GAT-2 and GAT-3 display greater sensitivity to the glial-selective drug β -alanine than does the previously cloned GAT-1, suggesting similarity to the transporter(s) characterized in glial cell cultures. However, a lack of identity with the pharmacologically defined glial-type transporter is demonstrated by the finding that guvacine, nipecotic acid, Tiagabine, hydroxynipecotic acid are much less potent inhibitors of GABA uptake at GAT-2 and GAT-3 than at the transporter present in glial cultures (13, 24, 47, 92). Additionally, these compounds are more potent in neuronal cultures (and at the previously cloned GAT-1) than at GAT-2 and GAT-3, which also distinguishes the newly cloned transporters from the neuronal GABA transporter (13, 24, 30, 47 and 92). Lastly, although GAT-2 and GAT-3 display similar sensitivity to a number of the inhibitors examined and show similar affinity for GABA itself, they can be distinguished by L-DABA, which displays higher potency at GAT-2 than at GAT-3. Interestingly, the potency of L-DABA at GAT-2 is similar to that of GAT-1 (Table 2), blurring the distinction between the newly cloned transporters and the neuronal-type transporter. This finding may indicate that a spectrum of GABA transport activities underlie the neuronal and glial profiles observed in tissue preparations. Lastly, the three cloned GABA transporters can also be distinguished by their differential dependence on external chloride: GAT-1 is the most chloride dependent, GAT-2 the least, and GAT-3 is intermediate in its sensitivity. The finding that GABA transport by GAT-2 and GAT-3 is not completely eliminated in chloride-free medium suggests that their mechanism of transport is fundamentally different from that of GAT-1.

It is somewhat surprising that the pharmacological profiles of GAT-2 and GAT-3 differ from those of previously characterized transporters in neuronal and glial cultures. One possible explanation is that the unique pharmacology of GAT-2 and GAT-3 reflects species differences, as the cloned transporters were obtained from a rat cDNA library, while mouse tissue was employed in many of the earlier studies (24, 47 and 92). This hypothesis gains validity from the finding that certain GABA uptake blockers are potent anti-convulsants in rats, but are ineffective in mice (113), although differences in drug metabolism or distribution have not been ruled out. A second possibility is that since neuronal and glial cultures are prepared from fetal or newborn animals, the discrepant results may reflect developmental changes in GABA transporters or peculiarities of Gila and neurons when maintained in cell culture. Alternatively, the two newly cloned transporters may in fact represent members of a novel class of transporters that have not been previously identified, perhaps due to their low abundance in cultured cells. This would suggest that further GABA transporters with pharmacological profiles consistent with those seen in neuronal and glial cultures remain to be cloned. Lastly, it should be pointed out that the pharmacological profiles of cloned transporters for serotonin (8,33), dopam-

ine (44,95), and norepinephrine (79), as well as GAT-1 are similar to those observed in brain homogenates, thus arguing that the unique properties of GAT-2 and GAT-3 are not the result of the heterologous expression system.

Despite the generally similar pharmacology of GAT-2 and GAT-3, their patterns of distribution are distinct. All three high-affinity GABA transporters are present in brain and retina, while only GAT-2 was detected in peripheral tissues. This finding is consistent with recent studies suggesting a role for GABA in liver (72), kidney (3,28) and other peripheral tissues (for review, ref. 23). Further distribution studies of GAT-2 and GAT-3 by in situ localization of transporter mRNAs in conjunction with immunocytochemistry will help to define the roles of these transporters in GABAergic transmission.

In conclusion, we now report the identification in mammalian brain of two novel high-affinity GABA transporters with unique pharmacological properties. These studies indicate previously unsuspected complexity in the regulation of GABAergic transmission, and provide the opportunity for the development of selective therapeutic agents to treat neurological and psychiatric disorders.

Cloning of Human High-Affinity GABA Transporters:

The use of human gene products in the process of drug development offers significant advantages over those of other species, which may not exhibit the same pharmacologic profiles. To facilitate this human-target based approach to drug design in the area of inhibitory amino acid transporters, we used the nucleotide sequences of the rat GAT-2 and GAT-3 cDNAs to clone the human homologues of each gene.

To obtain a cDNA clone encoding the human GAT-2 GABA transporter (hGAT-2) we used PCR primers based on the rat GAT-2 sequence to detect the presence of hGAT-2 in human cDNA libraries. PCR was carried out at a reduced annealing temperature to allow mismatches between rat and human sequences (see Experimental Procedures); amplified hGAT-2 sequences were detected by hybridization at low stringency with radiolabeled (randomly primed) rat GAT-2 cDNA. A human heart cDNA library (Stratagene) was identified and screened at low stringency with the same probe, resulting in isolation of a partial cDNA clone (hHE7a) containing the C-terminal portion of the coding region of hGAT-2. Using human sequence derived from this clone, a partial cDNA clone (hS3a) was isolated from a human striatum cDNA library (Stratagene) that provided additional sequence in the coding region. The hGAT-2 nucleotide sequence from these two clones and the deduced amino acid sequence based on translation of a long open reading frame is shown in FIGS. 14A–14B. The sequence includes 738 base pairs of coding region (246 amino acids) and 313 base pairs of 3' untranslated region. Comparison with the rat GAT-2 amino acid sequence reveals 90% identity over the region encoded by the clones, which includes predicted transmembrane domains 8–12 and the carboxy terminus of hGAT-2.

To obtain the nucleotide sequence of the human GAT-3 GABA transporter (hGAT-3), degenerate PCR primers were used to amplify transporter sequences from human cDNA libraries. Amplified hGAT-3 sequences were detected in the library by hybridization at low stringency with radiolabeled oligonucleotides representing the region of the rat GAT-3 cDNA that encodes a portion of the second extracellular loop. The human fetal brain library (Stratagene) identified by this approach was screened at high-stringency with the same probes; positive plaques were purified by successive screening at low stringency. Two cDNA clones were isolated

(hFB16a, hFB20a) which together comprise nearly the entire coding region of hGAT-3; the sequence of the remaining 7 base pairs was supplied by a genomic clone (hp28a) isolated from a human placental library. A vector comprising the complete coding sequence of hGAT-3 was constructed using appropriate fragments of these three clones, and is designated pcEXV-hGAT-3. The complete nucleotide sequence and predicted amino acid sequence of hGAT-3 are shown in FIGS. 15A–15D. In addition to 1896 base pairs of coding region, the sequence includes 5' and 3' untranslated sequence (34 and 61 base pairs, respectively). Translation of a long open reading frame predicts a protein of 632 amino acids that is 95% identical to the rat GAT-3 and contains 12 putative transmembrane domains. Methods similar to methods used to clone the human homologues of the mammalian GABA transporters can similarly be used to clone the human homologues of the mammalian taurine transporter.

The cloning and expression of the human GAT-2 and GAT-3 will allow comparison of pharmacological profiles with those of rat GABA transporters, and also provide a means for understanding and predicting the mechanism of action of GABA uptake inhibitors as human therapeutics. Recently several additional transporters have been cloned which exhibit significant sequence homology with previously cloned neurotransmitter transporters. cDNA and genomic clones representing the mouse homologues of GAT-1 were recently reported (52). In addition, a glycine transporter cDNA that is similar but not identical to that cloned by Smith et al. (98) was cloned from both rat (32) and mouse (52). A high-affinity L-proline transporter was reported by Freneau et al. (27), supporting a role for L-proline in excitatory neurotransmission. A rat cDNA identified as a choline transporter was reported by Mayser et al. (69). A taurine transporter cDNA was recently cloned from dog kidney cells (106) which is 90% identical to the rat taurine transporter amino acid sequence reported by Smith et al. (96). A cDNA encoding a mouse GABA transporter was recently cloned by Lopez-Corcuera et al. (59); the transporter encoded by this cDNA is 88% identical to the dog betaine transporter (110), and may represent the mouse homologue of that gene. Finally, a β -alanine-sensitive GABA transporter from rat brain has been cloned (19) that exhibits 100% amino acid identity with the rat GAT-3 sequence reported by Borden et al. (9).

Enhanced uptake of ^3H -GABA was observed with both transient and stable transfectants expressing hGAT-3. hGAT-3 displayed high affinity for GABA ($\text{IC}_{50} \approx 7 \mu\text{M}$) and for β -alanine ($\text{IC}_{50} \approx 58 \mu\text{M}$). Northern blot analysis of human total RNA reveals a ≈ 4 kb transcript which hybridized at high stringency with hGAT-3.

2. Taurine

Results and Discussion

Cloning of Mammalian Taurine Transporter:

We screened a rat brain cDNA library at low stringency with probes encoding the rat brain GABA transporter GAT-1 (31) in order to identify additional inhibitory amino acid transporter genes. Several clones were isolated which hybridized at low but not at high stringency with the GABA transporter probes. Characterization of the clones by DNA sequence analysis revealed that they represented a novel transporter sequence related to GAT-1. None of the clones contained the complete coding region of the putative transporter, and thus the library was rescreened at high stringency using oligonucleotides designed from the new sequence. A 2.5 kb cDNA clone (designated rB16a) was isolated which contained an open reading frame of 1863

base pairs encoding a protein of 621 amino acids (FIGS. 3A–3D). Comparison of this sequence with the rat GABA transporter cDNA revealed 58% nucleotide identity within the coding region. Comparison with sequences in Genbank and EMBL data bases demonstrated that the sequence was novel and that the most closely related sequence was the rat GABA transporter (31) followed by the human norepinephrine transporter (79). Subsequent comparisons to recently cloned transporters indicate that the most homologous sequences are two novel GABA transporters designated GAT-2 and GAT-3 (9) and the betaine transporter (110), which exhibit 62–64% nucleotide identity with rB16a.

The amino acid sequence deduced from the nucleotide sequence of rB16a is shown in FIGS. 5A–5B with a membrane topology similar to that proposed for the rat GABA transporter (31) and other cloned neurotransmitter transporters (8, 33, 44, 79 and 95). The translation product of rB16a is predicted to have a relative molecular mass of ~70,000 Daltons. Hydropathy analysis indicates the presence of 12 hydrophobic domains which may represent membrane spanning segments. Three potential sites for Asn-linked glycosylation are found in the extracellular loop between the third and fourth transmembrane domains. Alignment of the deduced amino acid sequence of rB16a with the rat GABA transporter (GAT-1; (31)) and the dog betaine transporter (110) revealed 50% and 58% amino acid identities, respectively (FIGS. 10A–10C). Comparison of rB16a with the glycine transporter (FIGS. 10A–10C; (98)) and the human norepinephrine transporter (79) also showed significant amino acid homology (41–45%), similar to that between GAT-1 and the norepinephrine transporter (46%). As predicted from nucleotide comparisons, the strongest amino acid homology (~61%) is with the GABA transporters GAT-2 and GAT-3 recently cloned from brain (9). In contrast, the sodium/glucose cotransporter (32), which shows a low degree of homology with cloned neurotransmitter transporters, displays only 21% amino acid identity with rB16a. These data suggested that the new sequence might encode an inhibitory amino acid transporter expressed in the brain. To explore this possibility, rB16a was placed in a mammalian expression vector, transfected into COS cells, and screened for transport of a variety of radiolabeled neurotransmitters and amino acids.

Pharmacological Characterization of Mammalian Taurine Transporter:

COS cells transiently transfected with rB16a (COS/rB16a) accumulated approximately 6-fold more [^3H]taurine than control, non-transfected cells (FIG. 11). Specific uptake represented greater than 95% of total uptake in transfected cells. In contrast, the uptake of [^3H]glutamate, [^3H]glycine, [^3H]5-HT, [^3H]dopamine, and [^3H]GABA was unaltered. Uptake of [^3H]taurine was not observed following mock transfection, indicating that the enhanced uptake was not the result of non-specific perturbation of the membrane. The transport of [^3H]taurine by COS/rB16a was decreased >95% when Na^+ was replaced by Li^+ , or when Cl^- was replaced by acetate (FIG. 11). In the absence of sodium or chloride, taurine transport in COS/rB21a decreased to levels below that of non-transfected controls, demonstrating that endogenous taurine transporter activity present in COS cells is also dependent on these ions. A similar ion dependence has been observed for taurine transport in vivo (36), as well as for the activity of other cloned neurotransmitter transporters such as those for GABA (31), glycine (95), and norepinephrine (79).

To determine the affinity of taurine for the cloned transporter, COS/rB16a was incubated with various concentrations of [^3H]taurine and the specific accumulation of

radioactivity was determined. Accumulation of [^3H]taurine was dose-dependent and reached saturation at higher concentrations (FIG. 12). Non-linear regression analysis of the data yielded the following values: $K_M=43\pm6\ \mu\text{M}$, and $V_{MAX}=0.96\pm0.27\ \text{nmoles/mg protein (mean}\pm\text{SEM, n=4 experiments)}$. The affinity of the cloned transporter for taurine is similar to that of high-affinity taurine transporters in both the central nervous system (56,111) and peripheral tissues (115) which exhibit K_M values from 10 to 60 μM . Taken together, these data indicate that rB16a encodes a saturable, high-affinity, sodium- and chloride-dependent taurine transporter.

To determine the pharmacological specificity of the cloned transporter, various agents were examined for their ability to inhibit the transport of [^3H]taurine by COS/rB16a (Table 15). As the endogenous taurine transporter in COS cells accounted for, on average, 16% of the total transport activity observed in transfected cells, we were concerned that this could influence results. Accordingly, we also examined the sensitivity of the endogenous taurine transporter present in non-transfected cells. As shown in Table 15, the pharmacologic properties of the cloned taurine transporter closely matched those of the endogenous transporter and thus did not lead to erroneous results.

The most potent inhibitors were taurine and hypotaurine, each of which inhibited specific [^3H]taurine uptake approximately 30–40% at 10 μM , 90% at 100 μM , and 100% at 1 mM. β -alanine was slightly less potent, inhibiting specific uptake 15%, 51%, and 96% at 10 μM , 100 μM , and 1 mM, respectively; the high potency of β -alanine as an inhibitor of taurine uptake is consistent with the finding that COS/rB16a showed a 6-fold increase in the specific uptake of [^3H] β -alanine (data not shown), essentially identical to the fold-increase observed with [^3H]taurine. The taurine analogue GES was also quite potent, inhibiting specific uptake of [^3H]taurine 11%, 45% and 92% at 10 μM , 100 μM and 1M, respectively. APSA and GABA both inhibited uptake approximately 10% and 40% at 100 μM and 1mM, respectively. The observations that GABA is a poor inhibitor of taurine uptake, and that transfection with rB16a did not result in enhanced uptake of [^3H]GABA (see above), are consistent with the previous report (65) that GABA is a weak non-competitive inhibitor of taurine uptake. Less than 10% inhibition of [^3H]taurine uptake was observed for the following compounds (each tested at 1mM): the structural analogues AEPA and MEA as well as the sulfur-containing amino acids cysteine and methionine (Table 15), and (data not shown) norepinephrine, dopamine, glutamate, glycine, serine, betaine, L-methionine, and α -methylaminoisobutyric acid (a substrate for amino acid transporter designated system A; (42)). Taken together, these results indicate that the taurine transporter encoded by rB16a is similar to the endogenous taurine transporter in COS cells (Table 15), as well as the endogenous taurine transporter(s) present in neural tissue (35), (see also ref. 36 and references therein).

It is of interest that sensitivity to β -alanine is shared by the two high-affinity GABA transporters recently cloned from rat brain (GAT-2 and GAT-3 (9)), which are even more closely related to the taurine transporter (62% amino acid identity) than to the neuronal-type GABA transporter GAT-1 (51%). β -alanine has been shown to activate an inward chloride current in spinal neurons (18,68) and is released in a calcium-dependent manner from several brain areas (42, 88), suggesting a role as an inhibitory neurotransmitter in the CNS. The similar sensitivities of the newly cloned GABA transporters (9) and the taurine transporter to β -alanine, combined with their sequence homologies, suggest that they

represent a subfamily of inhibitory neurotransmitter transporters. Despite these similarities, these transporters unexpectedly exhibit widely divergent affinities for GABA: GAT-2 and GAT-3 show the highest affinity ($K_m=10\text{ }\mu\text{M}$ (9)), while the affinity of the taurine transporter is extremely low ($\sim 1\text{ mM}$, Table 15). Interestingly, the dog betaine transporter (110), which displays a similar degree of homology to the members of this subfamily (ca. 60%), exhibits an intermediate affinity for GABA ($\sim 100\text{ }\mu\text{M}$). The finding that four structurally related transporters display overlapping substrate specificities for the neuroactive amino acids GABA and β -alanine suggests that multiple transporters may regulate the synaptic levels of these substances. This crossreactivity underscores the importance of understanding the action of therapeutic agents at both GABA and taurine transporters.

TABLE 15

Pharmacological Specificity of [³ H]taurine Uptake.			
Inhibitor ^a	Concentration	% Inhibition	
		control	rB16a
AEPA	1 mM	0 ± 0 (4)	3 ± 3 (5)
AMSA	1 mM	1 ± 1 (4)	7 ± 3 (4)
APSA	100 μM	7 ± 3 (4)	8 ± 4 (4)
β -alanine	1 mM	45 ± 3 (5)	36 ± 4 (5)
	10 μM	9 ± 2 (6)	15 ± 6 (6)
	100 μM	63 ± 3 (6)	51 ± 4 (10)
CSA	1 mM	97 ± 1 (4)	96 ± 1 (8)
	1 mM	2 ± 1 (4)	7 ± 5 (3)
	1 mM	4 ± 3 (3)	2 ± 2 (3)
GABA	10 μM	1 ± 1 (4)	9 ± 6 (4)
	100 μM	9 ± 4 (6)	10 ± 4 (10)
	1 mM	49 ± 2 (5)	44 ± 6 (8)
GES	10 μM	6 ± 3 (4)	11 ± 4 (4)
	100 μM	47 ± 3 (5)	45 ± 5 (5)
	1 mM	89 ± 1 (5)	92 ± 1 (6)
hypotaurine	10 μM	41 ± 3 (7)	26 ± 7 (7)
	100 μM	91 ± 1 (4)	84 ± 3 (4)
	1 mM	99 ± 1 (4)	100 ± 1 (4)
MEA	1 mM	1 ± 0 (3)	3 ± 3 (4)
methionine	1 mM	1 ± 1 (3)	1 ± 1 (3)
	10 μM	38 ± 5 (7)	29 ± 8 (5)
	100 μM	89 ± 2 (4)	83 ± 2 (5)
taurine	1 mM	100 ^b	100 ^b

^aNon-transfected COS-7 cells (control) or COS-7 cells transfected with rB16a were incubated for 10 minutes (37° C.) with 50 nM [³H]taurine and the indicated compounds. Data show percent displacement of specific [³H] taurine uptake (mean ± SEM; values in parentheses indicate number of experiments).

^bNon-specific uptake defined with 1 mM taurine.

Abbreviations: AEPA, 2-aminoethylphosphonic acid; AMSA, aminomethanesulfonic acid; APSA, 3-amino-1-propanesulfonic acid; CSA, cysteinesulfonic acid; GABA, gamma-aminobutyric acid; GES, guanidinoethanesulfonic acid; MEA, 2-mercaptoethylamine.

LIPOPHILIC GABA TRANSPORT INHIBITORS
SK&F 89976-A, TIAGABINE, CI-966, AND
NNC-711 ARE SELECTIVE FOR THE CLONED
GABA TRANSPORTER GAT-1

Molecular cloning has revealed a surprising diversity of GABA transporters. However, little is known regarding the relative contribution of the four cloned GABA transporters to GABAergic transmission. The lipophilic compounds SK&F 89976-A, Tiagabine, CI-966, and NNC-711 all display anticonvulsive activity in animals (Yunger et al., 1984; Swinyard et al., 1991; Nielsen et al., 1991; Taylor et al., 1990; Suzdak et al., 1992). Further, Tiagabine has demonstrated anti-seizure activity in patients in Phase II clinical trials (Chadwick et al., 1991), thus demonstrating the therapeutic utility of inhibiting GABA transport. Our finding that

these compounds are highly selective for GAT-1 demonstrates clearly that their anticonvulsive activity is mediated via actions at this site. It should be noted that GAT-1 is the predominant GABA transporter in the rat brain, accounting for approximately 85% of GABA transport when assayed in vitro.

Severe adverse effects were noted in human volunteers after administration of CI-966 (Sedman et al., 1990), but were not observed with Tiagabine (Chadwick et al., 1991). The finding that both compounds display a similar selectivity at the cloned GABA transporters suggests a common site of action in the brain. The untoward side-effects of CI-966 may have resulted from the high doses employed in the trials; alternatively, they may reflect interactions of either the parent compound, or a toxic metabolite, with sites distinct from GABA transporters.

Due to their specificity for GAT-1, the lipophilic compounds described above are important tools for the study of this transporter but offer little insight into the functions of the other cloned GABA transporters; future experiments will be aimed at the development of lipophilic ligands for these sites. Further study of the lipophilic GABA transport inhibitors should increase our understanding of GABA transporters and the role they play in regulating GABAergic activity, and may result in the development of novel therapeutic agents for anxiety, epilepsy, and other neuropsychiatric disorders.

Tissue Localization Studies of Mammalian Taurine Transporter:

To define the tissue distribution patterns of the taurine transporter, polymerase chain reaction (PCR) was used to detect the rB16a sequence in cDNA representing mRNA from seven different rat tissues. As a control, the distribution of the constitutively expressed protein cyclophilin was also examined. Radiolabeled oligonucleotides specific for rB16a were used to detect PCR products by hybridization. As shown in FIG. 13A, the taurine transporter was detectable in all tissues examined, including brain, retina, liver, kidney, heart, spleen, and pancreas, after 30 cycles of PCR. Cyclophilin was amplified to a similar extent from all the tissues (data not shown), demonstrating that adequate cDNA was present in each sample.

To evaluate both the abundance and the size of the mRNA encoding the taurine transporter, Northern blot analysis was carried out on poly A+ RNA isolated from the same rat tissues used for PCR analysis, with the addition of lung. As shown in FIG. 13B, a single ~6.2 kb transcript which hybridized with the taurine transporter cDNA probe was detected in brain, kidney, heart, spleen, and lung after an overnight exposure of the autoradiogram. After a 3-day exposure, bands of the same size were also visible in liver and pancreas (data not shown). Rehybridization of the blot with the cDNA encoding cyclophilin (12) confirmed that roughly equal amounts of RNA were present in each sample except that of retina, which was significantly degraded (data not shown). Thus, taurine transporter mRNA levels were highest in brain and lung, intermediate in kidney, heart, and spleen, and lowest in liver and pancreas. The abundance and pattern of distribution of the taurine transporter mRNA by Northern blot are consistent with data obtained using PCR (FIGS. 13A and 13B); further, the same size transcript is present in all tissues evaluated. These findings suggest that a single taurine transporter functions in both the brain and peripheral tissues; however, we can not exclude the existence of additional taurine transporters.

Taurine is abundant in the central nervous system and is involved in a variety of neural activities. Unlike classical

neurotransmitters, the effects of taurine are mediated both intra- and extracellularly. A major regulator of taurine levels, both within cells and in the synaptic cleft, is the transport of taurine across the plasma membrane. Our cloning of a high-affinity taurine transporter represents a critical step in defining the role of taurine in both neural and non-neural tissues, and in the development of therapeutic agents that alter taurine and GABA neurotransmission. In addition, the identification of a new member of the set of inhibitory amino acid transporters will aid in elucidating the molecular structure-function relationships within the transporter family.

GABA TRANSPORT IN NEURONAL AND GLIAL CELL CULTURES: CORRELATION OF PHARMACOLOGY AND mRNA LOCALIZATION

The application of molecular biology to the study of GABA transporters has provided considerable insight into this important class of synaptic proteins, but has also raised a number of questions. For example, it has not been clear which transporters are expressed in neurons and which in Gila, nor what role each plays in regulating synaptic transmission. Particularly confusing is the apparent lack of correspondence between the newly cloned transporters and the classically described glial transport systems. In an attempt to resolve these issues we have combined pharmacological studies with mRNA localization, employing primary cultures of neurons, and Type 1 and O-2A/Type 2 astrocytes. The results for each of these cell types will be discussed individually.

As expected from earlier studies, the majority of GABA transport activity in neuronal cultures has the properties of GAT-1, most notably high affinity for NNC-711 and ACHC, and low affinity for β -alanine. Consistent with these pharmacological results, we found that GAT-1 mRNA was abundant in neuronal cultures. These results are also consistent with in situ hybridization studies which demonstrate that GAT-1 mRNA is abundant in many GABAergic neurons in the rat brain (Rattray and Priestley 1993).

Interestingly, we observed that in neuronal cultures approximately 30% of GABA uptake is mediated by a non-GAT-1 mechanism, a finding not recognized by earlier investigators. The pharmacologic profile of this activity correlated well with GAT-2 and GAT-3, whose profiles are similar. However, Northern blot analysis revealed the presence in these cultures of mRNA for GAT-3 but not GAT-2, suggesting that GAT-3 mediates the uptake. The correlation with BGT-1 ($r=0.687$) was only slightly better than the correlation between GAT-3 and BGT-1 (0.503), suggesting that rBGT-1 makes only a minor contribution to the observed GABA transport. Consistent with this hypothesis, rBGT-1 mRNA was either absent, or present at very low levels in neuronal cultures. A role for GAT-3 in neuronal cultures is consistent with in situ hybridization studies which demonstrate that GAT-3 mRNA is present in rat brain neurons (Clark et al., 1992), though it is less abundant than GAT-1. Taken together, these data indicate clearly that neuronal GABA transport is heterogeneous, though it is not yet known whether both GABA transporters are expressed in individual neurons.

In contrast to neurons, Type 1 astrocytes transport only low levels of GABA and the transport is sensitive to β -alanine, in agreement with earlier autoradiographic studies (Reynolds and Herschkowitz, 1984). However, we found that the transport activity had low affinity for GABA and

high affinity for taurine, suggesting that uptake is mediated by TAUT. It is interesting that we have recently observed similar results with a variety of glial cell lines. Northern blots of Type 1 astrocytes demonstrated the presence of mRNA for TAUT and rBGT-1, but the absence of mRNA for GAT-1, GAT-2, and GAT-3. GABA transport activity best with TAUT, though the correlation was only modest ($r=0.523$). There was essentially no correlation with BGT-1, despite the relative abundance of rBGT-1 mRNA.

This discrepancy might reflect pharmacologic differences between rat and human BGT-1, as we have observed between human, mouse, and dog BGT-1; confirmation of this issue must await cloning of a full length cDNA for rBGT-1. Alternatively, we cannot rule out the existence in Type 1 astrocytes of an as-yet uncloned transporter. Nonetheless, the low level of [^{3} H]GABA transport in Type 1 astrocytes, and the low affinity for GABA, suggest that BGT-1 makes little contribution to GABA transport in these cells, despite the presence of rBGT-1 mRNA. It is possible that this discrepancy is an artifact of cell culture and that BGT-1 is expressed at high levels in vivo. A similar phenomenon has been reported in GH3 cells which have been shown to produce mRNA for the dopamine D2 receptor, although receptor binding is not detectable (Missale et al., 1991).

GABA transport in O-2A/Type 2 astrocytes differed considerably from that in Type 1 astrocytes, as expected from earlier autoradiographical studies. Previous investigations of GABA transport in astrocytes of the O-2A/Type 2 revealed these cells to have a "neuronal-like" pharmacology, based on their sensitivity to ACHC and insensitivity to β -alanine (Levi et al., 1983; Reynolds and Herschkowitz, 1984; Johnstone et al., 1986). However, precise interpretation of these data was complicated by the use of single concentrations of inhibitor and the qualitative nature of the assay (Reynolds and Herschkowitz, 1984; Johnstone et al., 1986), or the use of mixed astrocyte cultures (Levi et al., 1983). Our combined pharmacological and mRNA analyses establish unequivocally not only that GAT-1 is present in O-2A/Type 2 astrocyte cultures but that it is abundant in these cells, as it is in neurons. These results are in agreement with those of Mabeesh et al. (1992) who observed GAT-1-like immunoreactivity in cultured astrocytes with an O-2A/Type 2 morphology. Importantly, the data are also consistent with those of Radian et al. (1990), who observed GAT-1 immunoreactivity in glial processes in sections of rat brain.

We also observed that approximately 25% of GABA transport in O-2A/Type 2 astrocytes was resistant to NNC-711 and was thus mediated by a transporter distinct from GAT-1. The pharmacological profile correlated best with GAT-2 and GAT-3, and Northern blots revealed the presence in these cells of mRNA for both of these transporters. The levels of GAT-2 mRNA were greater than those for GAT-1, whereas GAT-3 mRNA was present at lower levels. Thus, despite quantitative differences, there is qualitative agreement between the pharmacology and mRNA localization. mRNA for rBGT-1 was also fairly abundant though the correlation with hBGT-1 ($r=0.685$) was only slightly greater than would be expected were the transport activity mediated solely by GAT-2 and/or GAT-3 (see Table 9). Taken together, the data suggest that the NNC-711-resistant uptake in O-2A/Type 2 astrocytes is mediated primarily by GAT-2 though GAT-3 and to a lesser extent rBGT-1, may also contribute. It is interesting to note that O-2A/Type 2 astrocytes exhibit the greatest heterogeneity in expression of GABA transporters, with all five present.

In brain aggregates, 85% of GABA uptake has a GAT-1-like pharmacology and GAT-1 mRNA is abundant. mRNA

for CAT-2, GAT-3, and BGT-1 are all present though surprisingly, these transporters together account for approximately 15% of total GABA transport, thus suggesting a mismatch between protein and mRNA levels.

Previous autoradiographic studies in which brain slices were incubated with [³H]GABA suggested that glial cells make only a minor contribution to overall GABA in vivo (Iversen and Bloom, 1972; Martin, 1976). This is consistent with our finding of low GABA uptake in Type 1 astrocytes but is in contrast to O-2A/Type 2 astrocytes, which avidly accumulate GABA. However, it has been suggested (Marriott and Wilkin, 1993) that Type 2 astrocytes correspond to reactive astrocytes which proliferate in response to brain injury, but which are rare in the normal (i.e., non-injured) brain. A similar situation has been demonstrated for nerve growth factor mRNA which is not present in adult Gila, but whose expression is high during development and following injury, as well as in actively growing astrocytes in vitro (Lu et al., 1991). The hypothesis that glial cells make only a minor contribution to GABA uptake in vivo is supported by in situ hybridization studies of transporter mRNAs which reveal little or no labeling in glial cells (Rattray and Priestley, 1993; Clark et al., 1992), despite heavy labeling in many populations of neurons. It is possible that GABA transporter mRNAs are present in glial cells but at low copy numbers, which are below the limits of detection by in situ hybridization. Amplification of the signal by in situ PCR may be required to visualize these transporters in glial cells. Alternatively, the apparent absence of GABA transporter mRNA in glial cells may be a technical problem relating to the small size of glial cells, since silver grains are more readily observed in large cells. It is clear that further experiments are required to resolve this critical issue.

Numerous animal studies (reviewed in Krogsgaard-Larsen et al., 1987) and more recently, clinical studies with human patients (Chadwick et al., 1991), indicate that inhibitors of GABA transport possess anti-convulsive activity, presumably by increasing GABAergic transmission. It has been argued that glial transporters are the preferred target since inhibition of neuronal transport would be expected to result in depletion of GABA in the nerve terminal; the net effect of this depletion would be an eventual decrease in GABAergic transmission (Krogsgaard-Larsen et al., 1987). However, the findings that 1. GAT-1 is not restricted to neurons; 2. neuronal transport appears to predominate over glial transport; and 3. β -alanine-sensitive transporters are observed in neurons, as well as Gila, suggest a re-evaluation of the role of neuronal and glial transporters in the treatment of convulsive disorders. It should be emphasized that the presence of GAT-1 in neurons and Gila precludes the development of a glial-(or neuronal) selective drug directed towards this site.

In conclusion, we have utilized a combined pharmacological and molecular biological approach to re-examine GABA transport in neuronal and glial cell cultures. The salient points of these studies may be summarized as follows: 1. GAT-1 mRNA is present in both neurons and O-2A/Type 2 astrocytes, and appears to account for the majority of GABA transport in both cell types; 2. GAT-3 mRNA is observed in neurons and O-2A/Type 2 astrocytes, though at levels below those of GAT-1 or GAT-2; 3. GAT-2 mRNA is present in O-2A/Type 2 cells, but is not detectable (by Northern blots) in neurons; 4. Type 1 astrocytes exhibit very low levels of GABA transport, and this activity has low affinity for GABA but high affinity for taurine. mRNAs for TAUT and BGT-1 are present in these cultures, whereas those for GAT-1, GAT-2 and GAT-3 are not detected; 5. the

pharmacologic data in glial cells can be explained by the presence of various combinations of the known cloned transporters, and no evidence was obtained for the existence of the classical "glial transporter". These findings provide new insight into the regulation of GABAergic transmission in the central nervous system and should aid in the development of novel therapeutic agents acting at GABA transporters.

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50 55 60

Asn Gly Gly Gly Ala Phe Phe Ile Pro Tyr Leu Ile Phe Leu Phe Thr
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Cys Gly Ile Pro Val Phe Phe Leu Glu Thr Ala Leu Gly Gln Tyr Thr
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Asn Gln Gly Gly Ile Thr Ala Trp Arg Lys Ile Cys Pro Ile Phe Glu
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Gly Ile Gly Tyr Ala Ser Gln Met Ile Val Ser Leu Leu Asn Val Tyr
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Tyr Ile Val Val Leu Ala Trp Ala Leu Phe Tyr Leu Phe Ser Ser Phe
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Thr Thr Asp Leu Pro Trp Gly Ser Cys Ser His Glu Trp Asn Thr Glu
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Glu Asn Ala Thr Ser Pro Val Ile Glu Phe Trp Glu Arg Arg Val Leu
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Lys Gly Val Lys Ser Thr Gly Lys Val Val Tyr Phe Thr Ala Thr Phe
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Gly Ala Ala Gln Gly Ile Gln Phe Tyr Leu Tyr Pro Asn Ile Thr Arg
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Ser Phe Ala Ile Cys Leu Gly Cys Leu Thr Ala Leu Gly Ser Tyr Asn
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Lys Tyr His Asn Asn Cys Tyr Arg Asp Cys Val Ala Leu Cys Ile Leu
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Gly Phe Met Ser Gln Glu Gln Gly Val Pro Ile Ser Glu Val Ala Glu
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Tyr	Asn	Asn	Asn	Cys	Tyr	Arg	Asp	Cys	Ile	Met	Leu	Cys	Cys	Leu	Asn
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Ser	Gly	Thr	Ser	Phe	Val	Ala	Gly	Phe	Ala	Ile	Phe	Ser	Val	Leu	Gly
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Phe	Met	Ala	Tyr	Glu	Gln	Gly	Val	Pro	Ile	Ala	Glu	Val	Ala	Glu	Ser
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Cys	Ile	Gly	Trp	Val	Tyr	Gly	Ser	Asn	Arg	Phe	Tyr	Asp	Asn	Ile	Glu
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Lys	Tyr	Lys	Pro	Leu	Lys	Tyr	Asn	Asn	Val	Tyr	Thr	Tyr	Pro	Ala	Trp
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Pro	Leu	Trp	Ile	Phe	Ile	Lys	Leu	Trp	Lys	Thr	Glu	Gly	Thr	Leu	Pro
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Ile Pro Tyr Phe Ile Phe Leu Phe Gly Ser Gly Leu Pro Val Phe Phe	
85 90 95	
Leu Glu Val Ile Ile Gly Gln Tyr Thr Ser Glu Gly Gly Ile Thr Cys	
100 105 110	
Trp Glu Lys Ile Cys Pro Leu Phe Ser Gly Ile Gly Tyr Ala Ser Ile	
115 120 125	
Val Ile Val Ser Leu Leu Asn Val Tyr Tyr Ile Val Ile Leu Ala Trp	
130 135 140	
Ala Thr Tyr Tyr Leu Phe Gln Ser Phe Gln Lys Asp Leu Pro Trp Ala	
145 150 155 160	
His Cys Asn His Ser Trp Asn Thr Pro Gln Cys Met Glu Asp Thr Leu	
165 170 175	
Arg Arg Asn Glu Ser His Trp Val Ser Leu Ser Ala Ala Asn Phe Thr	
180 185 190	
Ser Pro Val Ile Glu Phe Trp Glu Arg Asn Val Leu Ser Leu Ser Ser	
195 200 205	
Gly Ile Asp His Pro Gly Ser Leu Lys Trp Asp Leu Ala Leu Cys Leu	
210 215 220	
Leu Leu Val Trp Leu Val Cys Phe Phe Cys Ile Trp Lys Gly Val Arg	
225 230 235 240	
Ser Thr Gly Lys Val Val Tyr Phe Thr Ala Thr Phe Pro Phe Ala Met	
245 250 255	
Leu Leu Val Leu Leu Val Arg Gly Leu Thr Leu Pro Gly Ala Gly Glu	
260 265 270	
Gly Ile Lys Phe Tyr Leu Tyr Pro Asn Ile Ser Arg Leu Glu Asp Pro	
275 280 285	
Gln Val Trp Ile Asp Ala Gly Thr Gln Ile Phe Phe Ser Tyr Ala Ile	
290 295 300	
Cys Leu Gly Ala Met Thr Ser Leu Gly Ser Tyr Asn Lys Tyr Lys Tyr	
305 310 315 320	
Asn Ser Tyr Arg Asp Cys Met Leu Leu Gly Cys Leu Asn Ser Gly Thr	
325 330 335	

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Ser	Phe	Val	Ser	Gly	Phe	Ala	Ile	Phe	Ser	Ile	Leu	Gly	Phe	Met	Ala
			340					345					350		
Gln	Glu	Gln	Gly	Val	Asp	Ile	Ala	Asp	Val	Ala	Glu	Ser	Gly	Pro	Gly
		355					360					365			
Leu	Ala	Phe	Ile	Ala	Tyr	Pro	Lys	Ala	Val	Thr	Met	Met	Pro	Leu	Pro
	370					375					380				
Thr	Phe	Trp	Ser	Ile	Leu	Phe	Phe	Ile	Met	Leu	Leu	Leu	Leu	Gly	Leu
385					390					395					400
Asp	Ser	Gln	Phe	Val	Glu	Val	Glu	Gly	Gln	Ile	Thr	Ser	Leu	Val	Asp
				405					410					415	
Leu	Tyr	Pro	Ser	Phe	Leu	Arg	Lys	Gly	Tyr	Arg	Arg	Glu	Ile	Phe	Ile
			420					425					430		
Ala	Ile	Val	Cys	Ser	Ile	Ser	Tyr	Leu	Leu	Gly	Leu	Thr	Met	Val	Thr
		435					440					445			
Glu	Gly	Gly	Met	Tyr	Val	Phe	Gln	Leu	Phe	Asp	Tyr	Tyr	Ala	Ala	Ser
	450					455					460				
Gly	Val	Cys	Leu	Leu	Trp	Val	Ala	Phe	Phe	Glu	Cys	Phe	Val	Ile	Ala
465					470					475					480
Trp	Ile	Tyr	Gly	Gly	Asp	Asn	Leu	Tyr	Asp	Gly	Ile	Glu	Asp	Met	Ile
				485					490					495	
Gly	Tyr	Arg	Pro	Gly	Pro	Trp	Met	Lys	Tyr	Ser	Trp	Ala	Val	Ile	Thr
			500					505					510		
Pro	Ala	Leu	Cys	Val	Gly	Cys	Phe	Ile	Phe	Ser	Leu	Val	Lys	Tyr	Val
		515					520					525			
Pro	Leu	Thr	Tyr	Asn	Lys	Val	Tyr	Arg	Tyr	Pro	Asp	Trp	Ala	Ile	Gly
	530					535					540				
Leu	Gly	Trp	Gly	Leu	Ala	Leu	Ser	Ser	Met	Val	Cys	Ile	Pro	Leu	Val
545					550					555					560
Ile	Val	Ile	Leu	Leu	Cys	Arg	Thr	Glu	Gly	Pro	Leu	Arg	Val	Arg	Ile
				565					570					575	
Lys	Tyr	Leu	Ile	Thr	Pro	Arg	Glu	Pro	Asn	Arg	Trp	Ala	Val	Glu	Arg
			580					585					590		
Glu	Gly	Ala	Thr	Pro	Phe	His	Ser	Arg	Ala	Thr	Leu	Met	Asn	Gly	Ala
		595					600					605			
Leu	Met	Lys	Pro	Ser	His	Val	Ile	Val	Glu	Thr	Met	Met			
	610					615					620				

<210> SEQ ID NO 7
<211> LENGTH: 1051
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

ctggctttca	tcgcttacct	gcgggctgtg	gtgatgctgc	ccttctctcc	tctctgggcc	60
tgctgtttct	tcttcatggt	cgttctcctg	ggactggata	gccagtttgt	gtgtgtagaa	120
agcctggtga	cagcgctggt	ggacatgtac	cctcacgtgt	tccgcaagaa	gaaccggagg	180
gaagtcctca	tccttggagt	atctgtogtc	tccttccttg	tggggctgat	catgctcaca	240
gagggcgga	tgtacgtgtt	ccagctcttt	gactactatg	cggccagtgg	catgtgcctc	300
ctgttcgtgg	ccatcttcga	gtccctctgt	gtggcttggg	tttacggagc	caagcgcttc	360
tacgacaaca	tcgaagacat	gattgggtac	aggccatggc	ctcttatcaa	atactgttgg	420
ctcttcctca	caccagctgt	gtgcacagcc	acctttctct	tctccctgat	aaagtacact	480
ccgctgacct	acaacaagaa	gtacacgtac	cogtggtggg	gcgatgccct	gggctggctc	540

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ctggctctgt cctccatggt ctgcattcct gcctggagcc tctacagact cggaaccctc 600
aagggcccct tcagagagag aatccgtcag ctcatgtgcc cagccgagga cctgccccag 660
cggaacccag caggaccctc ggctcccgcc acccccagga cctcactgct cagactcaca 720
gagctagagt ctactgcta gggggcaggc ccttggtatg tgctgtgtg cctggccttg 780
gggatggctg tggaggaac gtggcagaag cagccccatg tgcttcctg cccccgacct 840
ggagtggata agacaagagg ggtattttgg agtccacctg ctgagctgga ggcctccac 900
tgcaactttt cagctcaggg gttgttgaac agatgtgaaa ggccagtgcc aagagtgtcc 960
ctctgagacc cttgggaagc tgggtggggg ctggtaggtg gggcgagact tgctggcttc 1020
gggccctctc atccttcatt ccattaaatc c 1051

<210> SEQ ID NO 8
<211> LENGTH: 246
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Leu Ala Phe Ile Ala Tyr Pro Arg Ala Val Val Met Leu Pro Phe Ser
1 5 10 15
Pro Leu Trp Ala Cys Cys Phe Phe Phe Met Val Val Leu Leu Gly Leu
20 25 30
Asp Ser Gln Phe Val Cys Val Glu Ser Leu Val Thr Ala Leu Val Asp
35 40 45
Met Tyr Pro His Val Phe Arg Lys Lys Asn Arg Arg Glu Val Leu Ile
50 55 60
Leu Gly Val Ser Val Val Ser Phe Leu Val Gly Leu Ile Met Leu Thr
65 70 75 80
Glu Gly Gly Met Tyr Val Phe Gln Leu Phe Asp Tyr Tyr Ala Ala Ser
85 90 95
Gly Met Cys Leu Leu Phe Val Ala Ile Phe Glu Ser Leu Cys Val Ala
100 105 110
Trp Val Tyr Gly Ala Lys Arg Phe Tyr Asp Asn Ile Glu Asp Met Ile
115 120 125
Gly Tyr Arg Pro Trp Pro Leu Ile Lys Tyr Cys Trp Leu Phe Leu Thr
130 135 140
Pro Ala Val Cys Thr Ala Thr Phe Leu Phe Ser Leu Ile Lys Tyr Thr
145 150 155 160
Pro Leu Thr Tyr Asn Lys Lys Tyr Thr Tyr Pro Trp Trp Gly Asp Ala
165 170 175
Leu Gly Trp Leu Leu Ala Leu Ser Ser Met Val Cys Ile Pro Ala Trp
180 185 190
Ser Leu Tyr Arg Leu Gly Thr Leu Lys Gly Pro Phe Arg Glu Arg Ile
195 200 205
Arg Gln Leu Met Cys Pro Ala Glu Asp Leu Pro Gln Arg Asn Pro Ala
210 215 220
Gly Pro Ser Ala Pro Ala Thr Pro Arg Thr Ser Leu Leu Arg Leu Thr
225 230 235 240
Glu Leu Glu Ser His Cys
245

<210> SEQ ID NO 9
<211> LENGTH: 1991
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens	
<400> SEQUENCE: 9	
agccgggccg gcgcacgagg cagccagcgc ggccatgacg gcggagaagg cgctgcccct	60
gggcaatggg aaggctgctg aggaggcgcg ggagtccgag gcgccgggtg gcggctgcag	120
cagcgggggc gcggcgcccg cgcgccaccc gcgcgtcaag cgcgacaagg cgggccacga	180
gcgcggccac tggaacaaca aggtggagtt cgtgctgagc gtggccgggg agatcattgg	240
gctgggcaac gtgtggcgct tcccctacct gtgtacaag aacggaggag gggcattcct	300
gattccctac gtggtgtttt ttatttgctg tggaattcct gtttttttcc tggagacagc	360
tctggggcag ttcacaagtg aaggtggcat tacgtgttgg aggaaagttt gccctttatt	420
tgaaggcatt ggctatgcaa cacaggtgat tgaggcccat ctgaatgtgt actacatcat	480
catcctggca tgggccatth tttacctgag caactgcttc actactgagc taccctgggc	540
tacctgtggg catgagtgga acacagagaa ttgtgtggag ttccagaaac tgaatgtgag	600
caactacagc catgtgtctc tgcagaatgc cacctcccct gtcatggagt tttgggagca	660
ccgggtcctg gccatctctg acgggatcga gcacatcggg aaccttcgct gggagctggc	720
cttgtgtctc ttggcagcct ggaccatctg ttactttctgt atctggaagg ggaccaagtc	780
tacaggaaag gttgtatacg tgactgcgac attcccctac atcatgctgc tgatcctcct	840
gatacgaggg gtcacgttgc ccggggcctc agagggcatc aagttctact tgtaccctga	900
cctctcccgg ctctccgacc ccaggtctg ggtagatgct ggaacgcaga tctttttctc	960
ctatgccatt tgcttgggct gtctgaccgc tctgggaagt tataacaatt ataacaaca	1020
ctgctacagg gactgcatca tgctctgttg cctgaacagc ggcaccagct tcgtggctgg	1080
gtttgccatc ttctcagtcc tgggttttat ggcgtacgag cagggggtag ccattgctga	1140
ggtggcagag tcaggccccg gcctggcctt tattgcgtac cccaaggcgg tcaccatgat	1200
gcctctctcc ccgctgtggg ccaccttggt cttcatgatg ctcatcttcc tgggcctgga	1260
cagccagttt gtgtgtgtgg aaagcctggg gaccgcctg gtggacatgt accccaaggt	1320
tttccggagg ggttaccggc gggagctgct catcctagcc ttgtctgtta tctcctatth	1380
tctgggcctc gtgatgttaa cagagggtggt catgtacatc ttccagctct ttgactccta	1440
tgccgccagt gggatgtgcc ttctcttcgt ggccatctth gagtgcactc gcatcggctg	1500
ggtgtatgga agcaaccggt tctatgataa cattgaagac atgattggct accggccacc	1560
gtcgtctcatt aagtgggtgt ggatgatcat gacctctggg atctgcgcgg ggatcttcat	1620
cttcttcttg atcaagtaca agccactcaa gtacaacaac atctacacct acccagcctg	1680
gggctatggc attggctggc tcatggccct gtctccatg ctctgcatcc cgctctggat	1740
ctgcatcaca gtgtggaaga cggaggggac actgcccgag aaactccaga agttgacgac	1800
ccccagcaca gatctgaaaa tgcggggcaa gcttgggggtg agcccacgga tggtgacagt	1860
taatgactgt gatgcaaac tcaagagtga cgggaccatc gcagccatca cagagaagga	1920
gacgcacttc tgagcggcca ccagccatct ggggctcttc ttcttttctt ccccccgtgt	1980
atgtaaatga a	1991
<210> SEQ ID NO 10	
<211> LENGTH: 632	
<212> TYPE: PRT	
<213> ORGANISM: Homo sapiens	
<400> SEQUENCE: 10	

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Met	Thr	Ala	Glu	Lys	Ala	Leu	Pro	Leu	Gly	Asn	Gly	Lys	Ala	Ala	Glu
1				5					10					15	
Glu	Ala	Arg	Glu	Ser	Glu	Ala	Pro	Gly	Gly	Gly	Cys	Ser	Ser	Gly	Gly
			20					25					30		
Ala	Ala	Pro	Ala	Arg	His	Pro	Arg	Val	Lys	Arg	Asp	Lys	Ala	Val	His
		35					40					45			
Glu	Arg	Gly	His	Trp	Asn	Asn	Lys	Val	Glu	Phe	Val	Leu	Ser	Val	Ala
	50					55					60				
Gly	Glu	Ile	Ile	Gly	Leu	Gly	Asn	Val	Trp	Arg	Phe	Pro	Tyr	Leu	Cys
65					70				75						80
Tyr	Lys	Asn	Gly	Gly	Gly	Ala	Phe	Leu	Ile	Pro	Tyr	Val	Val	Phe	Phe
				85					90					95	
Ile	Cys	Cys	Gly	Ile	Pro	Val	Phe	Phe	Leu	Glu	Thr	Ala	Leu	Gly	Gln
			100					105					110		
Phe	Thr	Ser	Glu	Gly	Gly	Ile	Thr	Cys	Trp	Arg	Lys	Val	Cys	Pro	Leu
		115					120					125			
Phe	Glu	Gly	Ile	Gly	Tyr	Ala	Thr	Gln	Val	Ile	Glu	Ala	His	Leu	Asn
	130					135					140				
Val	Tyr	Tyr	Ile	Ile	Ile	Leu	Ala	Trp	Ala	Ile	Phe	Tyr	Leu	Ser	Asn
145					150					155					160
Cys	Phe	Thr	Thr	Glu	Leu	Pro	Trp	Ala	Thr	Cys	Gly	His	Glu	Trp	Asn
				165				170						175	
Thr	Glu	Asn	Cys	Val	Glu	Phe	Gln	Lys	Leu	Asn	Val	Ser	Asn	Tyr	Ser
			180					185					190		
His	Val	Ser	Leu	Gln	Asn	Ala	Thr	Ser	Pro	Val	Met	Glu	Phe	Trp	Glu
		195					200					205			
His	Arg	Val	Leu	Ala	Ile	Ser	Asp	Gly	Ile	Glu	His	Ile	Gly	Asn	Leu
	210					215					220				
Arg	Trp	Glu	Leu	Ala	Leu	Cys	Leu	Leu	Ala	Ala	Trp	Thr	Ile	Cys	Tyr
225					230					235					240
Phe	Cys	Ile	Trp	Lys	Gly	Thr	Lys	Ser	Thr	Gly	Lys	Val	Val	Tyr	Val
				245					250					255	
Thr	Ala	Thr	Phe	Pro	Tyr	Ile	Met	Leu	Leu	Ile	Leu	Leu	Ile	Arg	Gly
			260					265					270		
Val	Thr	Leu	Pro	Gly	Ala	Ser	Glu	Gly	Ile	Lys	Phe	Tyr	Leu	Tyr	Pro
		275					280					285			
Asp	Leu	Ser	Arg	Leu	Ser	Asp	Pro	Gln	Val	Trp	Val	Asp	Ala	Gly	Thr
	290					295					300				
Gln	Ile	Phe	Phe	Ser	Tyr	Ala	Ile	Cys	Leu	Gly	Cys	Leu	Thr	Ala	Leu
305					310					315					320
Gly	Ser	Tyr	Asn	Asn	Tyr	Asn	Asn	Asn	Cys	Tyr	Arg	Asp	Cys	Ile	Met
				325					330					335	
Leu	Cys	Cys	Leu	Asn	Ser	Gly	Thr	Ser	Phe	Val	Ala	Gly	Phe	Ala	Ile
			340					345					350		
Phe	Ser	Val	Leu	Gly	Phe	Met	Ala	Tyr	Glu	Gln	Gly	Val	Pro	Ile	Ala
		355					360					365			
Glu	Val	Ala	Glu	Ser	Gly	Pro	Gly	Leu	Ala	Phe	Ile	Ala	Tyr	Pro	Lys
	370					375					380				
Ala	Val	Thr	Met	Met	Pro	Leu	Ser	Pro	Leu	Trp	Ala	Thr	Leu	Phe	Phe
385					390					395					400
Met	Met	Leu	Ile	Phe	Leu	Gly	Leu	Asp	Ser	Gln	Phe	Val	Cys	Val	Glu
				405					410					415	

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Ser	Leu	Val	Thr	Ala	Val	Val	Asp	Met	Tyr	Pro	Lys	Val	Phe	Arg	Arg
			420					425					430		
Gly	Tyr	Arg	Arg	Glu	Leu	Leu	Ile	Leu	Ala	Leu	Ser	Val	Ile	Ser	Tyr
		435					440					445			
Phe	Leu	Gly	Leu	Val	Met	Leu	Thr	Glu	Gly	Gly	Met	Tyr	Ile	Phe	Gln
	450					455					460				
Leu	Phe	Asp	Ser	Tyr	Ala	Ala	Ser	Gly	Met	Cys	Leu	Leu	Phe	Val	Ala
465					470					475					480
Ile	Phe	Glu	Cys	Ile	Cys	Ile	Gly	Trp	Val	Tyr	Gly	Ser	Asn	Arg	Phe
			485						490					495	
Tyr	Asp	Asn	Ile	Glu	Asp	Met	Ile	Gly	Tyr	Arg	Pro	Pro	Ser	Leu	Ile
		500						505					510		
Lys	Trp	Cys	Trp	Met	Ile	Met	Thr	Pro	Gly	Ile	Cys	Ala	Gly	Ile	Phe
		515					520					525			
Ile	Phe	Phe	Leu	Ile	Lys	Tyr	Lys	Pro	Leu	Lys	Tyr	Asn	Asn	Ile	Tyr
	530					535					540				
Thr	Tyr	Pro	Ala	Trp	Gly	Tyr	Gly	Ile	Gly	Trp	Leu	Met	Ala	Leu	Ser
545					550					555					560
Ser	Met	Leu	Cys	Ile	Pro	Leu	Trp	Ile	Cys	Ile	Thr	Val	Trp	Lys	Thr
			565						570					575	
Glu	Gly	Thr	Leu	Pro	Glu	Lys	Leu	Gln	Lys	Leu	Thr	Thr	Pro	Ser	Thr
			580					585					590		
Asp	Leu	Lys	Met	Arg	Gly	Lys	Leu	Gly	Val	Ser	Pro	Arg	Met	Val	Thr
		595					600					605			
Val	Asn	Asp	Cys	Asp	Ala	Lys	Leu	Lys	Ser	Asp	Gly	Thr	Ile	Ala	Ala
	610					615					620				
Ile	Thr	Glu	Lys	Glu	Thr	His	Phe								
625					630										

<210> SEQ ID NO 11
<211> LENGTH: 599
<212> TYPE: PRT
<213> ORGANISM: Rattus norvegicus

<400> SEQUENCE: 11

Met	Ala	Thr	Asp	Asn	Ser	Lys	Val	Ala	Asp	Gly	Gln	Ile	Ser	Thr	Glu
1				5					10					15	
Val	Ser	Glu	Ala	Pro	Val	Ala	Ser	Asp	Lys	Pro	Lys	Thr	Leu	Val	Val
			20					25					30		
Lys	Val	Gln	Lys	Lys	Ala	Gly	Asp	Leu	Pro	Asp	Arg	Asp	Thr	Trp	Lys
		35					40					45			
Gly	Arg	Phe	Asp	Phe	Leu	Met	Ser	Cys	Val	Gly	Tyr	Ala	Ile	Gly	Leu
	50					55					60				
Gly	Asn	Val	Trp	Arg	Phe	Pro	Tyr	Leu	Cys	Gly	Lys	Asn	Gly	Gly	Gly
65					70					75					80
Ala	Phe	Leu	Ile	Pro	Tyr	Phe	Leu	Thr	Leu	Ile	Phe	Ala	Gly	Val	Pro
				85					90					95	
Leu	Phe	Leu	Leu	Glu	Cys	Ser	Leu	Gly	Gln	Tyr	Thr	Ser	Ile	Gly	Gly
			100					105					110		
Leu	Gly	Val	Trp	Lys	Leu	Ala	Pro	Met	Phe	Lys	Gly	Val	Gly	Leu	Ala
		115					120					125			
Ala	Ala	Val	Leu	Ser	Phe	Trp	Leu	Asn	Ile	Tyr	Tyr	Ile	Val	Ile	Ile
	130					135					140				
Ser	Trp	Ala	Ile	Tyr	Tyr	Leu	Tyr	Asn	Ser	Phe	Thr	Thr	Thr	Leu	Pro
145					150					155					160

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Trp	Lys	Gln	Cys	Asp	Asn	Pro	Trp	Asn	Thr	Asp	Arg	Cys	Phe	Ser	Asn
				165					170					175	
Tyr	Ser	Leu	Val	Asn	Thr	Thr	Asn	Met	Thr	Ser	Ala	Val	Val	Glu	Phe
			180					185					190		
Trp	Glu	Arg	Asn	Met	His	Gln	Met	Thr	Asp	Gly	Leu	Asp	Lys	Pro	Gly
		195					200					205			
Gln	Ile	Arg	Trp	Pro	Leu	Ala	Ile	Thr	Leu	Ala	Ile	Ala	Trp	Val	Leu
	210					215					220				
Val	Tyr	Phe	Cys	Ile	Trp	Lys	Gly	Val	Gly	Trp	Thr	Gly	Lys	Val	Val
225					230				235						240
Tyr	Phe	Ser	Ala	Thr	Tyr	Pro	Tyr	Ile	Met	Leu	Ile	Ile	Leu	Phe	Phe
				245					250					255	
Arg	Gly	Val	Thr	Leu	Pro	Gly	Ala	Lys	Glu	Gly	Ile	Leu	Phe	Tyr	Ile
			260					265					270		
Thr	Pro	Asn	Phe	Arg	Lys	Leu	Ser	Asp	Ser	Glu	Val	Trp	Leu	Asp	Ala
		275					280					285			
Ala	Thr	Gln	Ile	Phe	Phe	Ser	Tyr	Gly	Leu	Gly	Leu	Gly	Ser	Leu	Ile
	290					295					300				
Ala	Leu	Gly	Ser	Tyr	Asn	Ser	Phe	His	Asn	Asn	Val	Tyr	Arg	Asp	Ser
305					310					315					320
Ile	Ile	Val	Cys	Cys	Ile	Asn	Ser	Cys	Thr	Ser	Met	Phe	Ala	Gly	Phe
			325						330					335	
Val	Ile	Phe	Ser	Ile	Val	Gly	Phe	Met	Ala	His	Val	Thr	Lys	Arg	Ser
			340					345					350		
Ile	Ala	Asp	Val	Ala	Ala	Ser	Gly	Pro	Gly	Leu	Ala	Phe	Leu	Ala	Tyr
	355						360					365			
Pro	Glu	Ala	Val	Thr	Gln	Leu	Pro	Ile	Ser	Pro	Leu	Trp	Ala	Ile	Leu
	370					375					380				
Phe	Phe	Ser	Met	Leu	Leu	Met	Leu	Gly	Ile	Asp	Ser	Gln	Phe	Cys	Thr
385					390					395					400
Val	Glu	Gly	Phe	Ile	Thr	Ala	Leu	Val	Asp	Glu	Tyr	Pro	Arg	Leu	Leu
			405						410					415	
Arg	Asn	Arg	Arg	Glu	Leu	Phe	Ile	Ala	Ala	Val	Cys	Ile	Val	Ser	Tyr
			420					425					430		
Leu	Ile	Gly	Leu	Ser	Asn	Ile	Thr	Gln	Gly	Gly	Ile	Tyr	Val	Phe	Lys
	435						440					445			
Leu	Phe	Asp	Tyr	Tyr	Ser	Ala	Ser	Gly	Met	Ser	Leu	Leu	Phe	Leu	Val
	450					455					460				
Phe	Phe	Glu	Cys	Val	Ser	Ile	Ser	Trp	Phe	Tyr	Gly	Val	Asn	Arg	Phe
465					470					475					480
Tyr	Asp	Asn	Ile	Gln	Glu	Met	Val	Gly	Ser	Arg	Pro	Cys	Ile	Trp	Trp
			485					490						495	
Lys	Leu	Cys	Trp	Ser	Phe	Phe	Thr	Pro	Ile	Ile	Val	Ala	Gly	Val	Phe
			500					505					510		
Leu	Phe	Ser	Ala	Val	Gln	Met	Thr	Pro	Leu	Thr	Met	Gly	Ser	Tyr	Val
		515					520					525			
Phe	Pro	Lys	Trp	Gly	Gln	Gly	Val	Gly	Trp	Leu	Met	Ala	Leu	Ser	Ser
	530					535					540				
Met	Val	Leu	Ile	Pro	Gly	Tyr	Met	Ala	Tyr	Met	Phe	Leu	Thr	Leu	Lys
545					550					555					560
Gly	Ser	Leu	Lys	Gln	Arg	Leu	Gln	Val	Met	Ile	Gln	Pro	Ser	Glu	Asp
				565					570					575	

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Ile	Val	Arg	Pro	Glu	Asn	Gly	Pro	Glu	Gln	Pro	Gln	Ala	Gly	Ser	Ser	
			580					585					590			
Ala	Ser	Lys	Glu	Ala	Tyr	Ile										
		595														
<210> SEQ ID NO 12																
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Trp	Leu	Pro	Glu	Glu	Gly	Glu	Lys	Leu	Asp	Gln	Glu	Gly	Glu	Asp	Gln	
			20					25					30			
Val	Lys	Asp	Arg	Gly	Gln	Trp	Thr	Asn	Lys	Met	Glu	Phe	Val	Leu	Ser	
		35					40					45				
Val	Ala	Gly	Glu	Ile	Ile	Gly	Leu	Gly	Asn	Val	Trp	Arg	Phe	Pro	Tyr	
	50					55					60					
Leu	Cys	Tyr	Lys	Asn	Gly	Gly	Gly	Ala	Phe	Phe	Ile	Pro	Tyr	Phe	Ile	
65					70					75					80	
Phe	Phe	Phe	Thr	Cys	Gly	Ile	Pro	Val	Phe	Phe	Leu	Glu	Val	Ala	Leu	
				85					90					95		
Gly	Gln	Tyr	Thr	Ser	Gln	Gly	Ser	Val	Thr	Ala	Trp	Arg	Lys	Ile	Cys	
			100					105					110			
Pro	Leu	Leu	Gln	Gly	Ile	Gly	Leu	Ala	Ser	Val	Val	Ile	Glu	Ser	Tyr	
	115						120					125				
Leu	Asn	Ile	Tyr	Tyr	Ile	Ile	Ile	Leu	Ala	Trp	Ala	Leu	Phe	Tyr	Leu	
130					135						140					
Phe	Ser	Ser	Phe	Thr	Ser	Glu	Leu	Pro	Trp	Thr	Thr	Cys	Thr	Asn	Thr	
145					150					155					160	
Trp	Asn	Thr	Glu	His	Cys	Met	Asp	Phe	Leu	Asn	His	Ser	Gly	Ala	Arg	
				165					170					175		
Thr	Ala	Thr	Ser	Ser	Glu	Asn	Phe	Thr	Ser	Pro	Val	Met	Glu	Phe	Trp	
			180					185					190			
Glu	Arg	Arg	Val	Leu	Gly	Ile	Thr	Ser	Gly	Ile	His	Asp	Leu	Gly	Ala	
	195						200				205					
Leu	Arg	Trp	Glu	Leu	Ala	Leu	Cys	Leu	Leu	Leu	Ala	Trp	Leu	Ile	Cys	
210					215						220					
Tyr	Phe	Cys	Ile	Trp	Lys	Gly	Val	Lys	Thr	Thr	Gly	Lys	Val	Val	Tyr	
225					230					235					240	
Phe	Thr	Ala	Thr	Phe	Pro	Tyr	Leu	Met	Leu	Val	Ile	Leu	Leu	Ile	Arg	
			245					250						255		
Gly	Ile	Thr	Leu	Pro	Gly	Ala	Tyr	Gln	Gly	Val	Ile	Tyr	Tyr	Leu	Lys	
			260					265					270			
Pro	Asp	Leu	Leu	Arg	Leu	Lys	Asp	Pro	Gln	Val	Trp	Met	Asp	Ala	Gly	
	275						280					285				
Thr	Gln	Ile	Phe	Phe	Ser	Phe	Ala	Ile	Cys	Gln	Gly	Cys	Leu	Thr	Ala	
290						295					300					
Leu	Gly	Ser	Tyr	Asn	Lys	Tyr	His	Asn	Asn	Cys	Tyr	Arg	Asp	Ser	Ile	
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Ala	Leu	Cys	Phe	Leu	Asn	Ser	Ala	Thr	Ser	Phe	Ala	Ala	Gly	Phe	Val	
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Val	Phe	Ser	Ile	Leu	Gly	Phe	Met	Ala	Gln	Glu	Gln	Gly	Leu	Pro	Ile	
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Ser Glu Val Ala Glu Ser Gly Pro Gly Leu Ala Phe Ile Ala Phe Pro
355 360 365

Lys Ala Val Thr Met Met Pro Leu Ser Gln Leu Trp Ser Cys Leu Phe
370 375 380

Phe Ile Met Leu Ile Phe Leu Gly Leu Asp Ser Gln Phe Val Cys Val
385 390 395 400

Glu Cys Leu Val Thr Ala Ser Met Asp Met Phe Pro Ser Gln Leu Arg
405 410 415

Lys Ser Gly Arg Arg Glu Leu Leu Ile Leu Ala Ile Ala Val Phe Cys
420 425 430

Tyr Leu Ala Gly Leu Phe Leu Val Thr Glu Gly Gly Met Tyr Ile Phe
435 440 445

Gln Leu Phe Asp Tyr Tyr Ala Ser Ser Gly Ile Cys Leu Leu Phe Leu
450 455 460

Ala Met Phe Glu Val Ile Cys Ile Ser Trp Val Tyr Gly Ala Asp Arg
465 470 475 480

Phe Tyr Asp Asn Ile Glu Asp Met Ile Gly Tyr Arg Pro Trp Pro Leu
485 490 495

Val Lys Ile Ser Trp Leu Phe Leu Thr Pro Gly Leu Cys Leu Ala Thr
500 505 510

Phe Leu Phe Ser Leu Ser Gln Tyr Thr Pro Leu Lys Tyr Asn Asn Ile
515 520 525

Tyr Val Tyr Pro Pro Trp Gly Tyr Ser Ile Gly Trp Phe Leu Ala Leu
530 535 540

Ser Ser Met Ile Cys Val Pro Leu Phe Val Ile Ile Thr Leu Leu Lys
545 550 555 560

Thr Arg Gly Ser Phe Lys Lys Arg Leu Arg Gln Leu Thr Thr Pro Asp
565 570 575

Pro Ser Leu Pro Gln Pro Lys Gln His Leu Tyr Leu Asp Gly Gly Thr
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595 600 605

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<213> ORGANISM: Rattus norvegicus

<400> SEQUENCE: 13

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Gly Ala Val Pro Ser Glu Ala Thr Lys Lys Asp Gln Asn Leu Thr Arg
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35 40 45

Ala Val Gly Leu Gly Asn Val Trp Arg Phe Pro Tyr Leu Cys Tyr Arg
50 55 60

Asn Gly Gly Gly Ala Phe Met Phe Pro Tyr Phe Ile Met Leu Val Phe
65 70 75 80

Cys Gly Ile Pro Leu Phe Phe Met Glu Leu Ser Phe Gly Gln Phe Ala
85 90 95

Ser Gln Gly Cys Leu Gly Val Trp Arg Ile Ser Pro Met Phe Lys Gly

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100						105						110					
Val	Gly	Tyr	Gly	Met	Met	Val	Val	Ser	Thr	Tyr	Ile	Gly	Ile	Tyr	Tyr		
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Asn	Val	Val	Ile	Cys	Ile	Ala	Phe	Tyr	Tyr	Phe	Phe	Ser	Ser	Met	Thr		
	130					135					140						
His	Val	Leu	Pro	Trp	Ala	Tyr	Cys	Asn	Asn	Pro	Trp	Asn	Thr	Pro	Asp		
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Cys	Ala	Gly	Val	Leu	Asp	Ala	Ser	Asn	Leu	Thr	Asn	Gly	Ser	Arg	Pro		
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Thr	Ala	Leu	Ser	Gly	Asn	Leu	Ser	His	Leu	Phe	Asn	Tyr	Thr	Leu	Gln		
			180					185					190				
Arg	Thr	Ser	Pro	Ser	Glu	Glu	Tyr	Trp	Arg	Leu	Tyr	Val	Leu	Lys	Leu		
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Ser	Asp	Asp	Ile	Gly	Asp	Phe	Gly	Glu	Val	Arg	Leu	Pro	Leu	Leu	Gly		
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Cys	Leu	Gly	Val	Ser	Trp	Val	Val	Val	Phe	Leu	Cys	Leu	Ile	Arg	Gly		
225					230					235					240		
Val	Lys	Ser	Ser	Gly	Lys	Val	Val	Tyr	Phe	Thr	Ala	Thr	Phe	Pro	Tyr		
				245					250					255			
Val	Val	Leu	Thr	Ile	Leu	Phe	Val	Arg	Gly	Val	Thr	Leu	Glu	Gly	Ala		
			260					265					270				
Phe	Thr	Gly	Ile	Met	Tyr	Tyr	Leu	Thr	Pro	Lys	Trp	Asp	Lys	Ile	Leu		
		275					280					285					
Glu	Ala	Lys	Val	Trp	Gly	Asp	Ala	Ala	Ser	Gln	Ile	Phe	Tyr	Ser	Leu		
	290					295					300						
Gly	Cys	Ala	Trp	Gly	Gly	Leu	Ile	Thr	Met	Ala	Ser	Tyr	Asn	Lys	Phe		
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His	Asn	Asn	Cys	Tyr	Arg	Asp	Ser	Val	Ile	Ile	Ser	Ile	Thr	Asn	Cys		
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Ala	Thr	Ser	Val	Tyr	Ala	Gly	Phe	Val	Ile	Phe	Ser	Ile	Leu	Gly	Phe		
			340					345					350				
Met	Ala	Asn	His	Leu	Gly	Val	Asp	Val	Ser	Arg	Val	Ala	Asp	His	Gly		
		355					360					365					
Pro	Gly	Leu	Ala	Phe	Val	Ala	Tyr	Pro	Glu	Ala	Leu	Thr	Leu	Leu	Pro		
	370					375					380						
Ile	Ser	Pro	Leu	Trp	Ser	Leu	Leu	Phe	Phe	Phe	Met	Leu	Ile	Leu	Leu		
385					390					395					400		
Gly	Leu	Gly	Thr	Gln	Phe	Cys	Leu	Leu	Glu	Thr	Leu	Val	Thr	Ala	Ile		
				405					410					415			
Val	Asp	Glu	Val	Gly	Asn	Glu	Trp	Ile	Leu	Gln	Lys	Lys	Thr	Tyr	Val		
			420					425					430				
Thr	Leu	Gly	Val	Ala	Val	Ala	Gly	Phe	Leu	Leu	Gly	Ile	Pro	Leu	Thr		
		435					440					445					
Ser	Gln	Ala	Gly	Ile	Tyr	Trp	Leu	Leu	Leu	Met	Asp	Asn	Tyr	Ala	Ala		
	450					455					460						
Ser	Phe	Ser	Leu	Val	Val	Ile	Ser	Cys	Ile	Met	Cys	Val	Ser	Ile	Met		
465					470					475					480		
Tyr	Ile	Tyr	Gly	His	Arg	Asn	Tyr	Phe	Gln	Asp	Ile	Gln	Met	Met	Leu		
				485					490					495			
Gly	Phe	Pro	Pro	Pro	Leu	Phe	Phe	Gln	Ile	Cys	Trp	Arg	Phe	Val	Ser		
			500					505					510				
Pro	Thr	Ile	Ile	Phe	Phe	Ile	Leu	Ile	Phe	Thr	Val	Ile	Gln	Tyr	Arg		
		515					520					525					

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Pro Ile Thr Tyr Asn His Tyr Gln Tyr Pro Gly Trp Ala Val Ala Ile
530 535 540

Gly Phe Leu Met Ala Leu Ser Ser Val Ile Cys Ile Pro Leu Tyr Ala
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Leu Phe Gln Leu Cys Arg Thr Asp Gly Asp Thr Leu Leu Gln Arg Leu
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Lys Asn Ala Thr Lys Pro Ser Arg Asp Trp Gly Pro Ala Leu Leu Glu
580 585 590

His Arg Thr Gly Arg Tyr Ala Pro Thr Thr Thr Pro Ser Pro Glu Asp
595 600 605

Gly Phe Glu Val Gln Pro Leu His Pro Asp Lys Ala Gln Ile Pro Ile
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Val Gly Ser Asn Gly Ser Ser Arg Leu Gln Asp Ser Arg Ile
625 630 635

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gaacttgatg ccttccgagg caccc 25

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What is claimed is:

1. An isolated nucleic acid molecule encoding a rat GABA transporter having an amino acid sequence selected from the group consisting of the amino acid sequence shown in Seq. I.D. No. 2 and the amino acid sequence shown in Seq. I.D. No. 4.

2. An isolated nucleic acid molecule encoding a human GABA transporter having an amino acid sequence selected from the group consisting of the amino acid sequence shown in Seq. I.D. No. 8 and the amino acid sequence shown in Seq. I.D. No. 10.

3. The isolated nucleic acid molecule of either of claim 1 or 2, wherein the nucleic acid molecule is a DNA molecule.

4. The DNA molecule of claim 3, wherein the DNA molecule is a cDNA molecule.

5. A vector comprising the DNA molecule of claim 3.

6. The vector of claim 5, wherein the vector is a plasmid.

7. The vector of claim 5 adapted for expression in a bacterial cell which comprises regulatory elements necessary for expression of the DNA in the bacterial cell so located relative to the DNA encoding the transporter as to permit expression thereof.

8. The vector of claim 5 adapted for expression in a yeast cell which comprises regulatory elements necessary for expression of the DNA in the yeast cell so located relative to the DNA encoding the transporter as to permit expression thereof.

9. The vector of claim 5 adapted for expression in a mammalian cell which comprises regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding the transporter as to permit expression thereof.

10. The plasmid of claim 6 adapted for expression in a mammalian cell which comprises regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding the GABA transporter as to permit expression thereof.

11. A plasmid designated pEVJB-rB14b (ATCC Accession No. 75203).

12. A plasmid designated pEVJB-rB8b (ATCC Accession No. 75201).

13. A plasmid designated pcEXV-hGAT-3 (ATCC Accession No. 75324).

14. A plasmid designated pBluescript hHE7a (ATCC Accession No. 75322).

15. A plasmid designated pBluescript hS3a (ATCC Accession No. 75323).

16. A mammalian cell comprising the plasmid of claim 10.

17. The mammalian cell of claim 16, wherein the mammalian cell is a Cos7 cell.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,225,115 B1
DATED : May 1, 2001
INVENTOR(S) : Kelli E. Smith et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 121,

Line 64, "caciprises" should be -- comprises --

Line 66, "tzhe" should be -- the --

Signed and Sealed this

Eighteenth Day of March, 2003

A handwritten signature in black ink, appearing to read "James E. Rogan", with a long horizontal stroke extending from the bottom of the signature.

JAMES E. ROGAN
Director of the United States Patent and Trademark Office